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Journal of DENTAL RESEARCH

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This *Journal* is dedicated to the dissemination of knowledge pertaining to the oral cavity, its contents and contiguous parts, and its relation to and effect on the total human organism. The *Journal* publishes original investigations in these and related fields. Articles are accepted only if they are contributed solely to this *Journal*.

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Editorial Views and News

The 38th annual meeting of the IADR recently held in Chicago portrayed many features of a rapidly growing research society.

1960 meeting data.—Registration totaled 820, which was higher than at the last Chicago meeting in 1955, when officially it amounted to 612. Elected to regular membership at this time was the somewhat enormous number of 156 individuals. Of these, 58 registered for the meeting. Other registrants included 323 members; 56 from the Dental Materials Group; 292 non-members; 65 dental students; and 26 guests.

At the meeting two excellent symposia were arranged on one evening, one by Joseph C. Muhler entitled "Mechanism of Fluoride Action" and the other by Edward V. Zegarelli on "Clinical Research."

During this 3½-day meeting, from March 17 to March 20, a total of 261 papers was actually presented, although 266 were printed in the program, with 53 additional papers listed for the Dental Materials Group. Yet many more papers were received by the program committee, some as late as the week of the meeting.

To emphasize the longevity of the Journal, an exhibit, prepared by the editorial office, was displayed in the exhibit room, its central theme being that the *Journal of Dental Research* has entered its fifth decade. The exhibit's white and sky-blue façade resembled the colors of the new *Journal* cover and pointed out that during the *Journal's* 41 years of existence 2,381 manuscripts have been published, almost half of which appeared in the *Journal* in the last 10 years.

The high point of every annual IADR meeting is the banquet. It was at the Chicago meeting of 5 years ago that the present editor, then chairman of local arrangements, arranged for the banquet after a lapse of several years. At this recent banquet on March 19 the President-elect, Holmes T. Knighton, presented a brief but stimulating paper on "An Evaluation of Clinical Research in Dentistry." This Presidential Inaugural Address will be published in a future issue along with the Proceedings of the meeting.

The presentation of awards at the banquet has now become a notable annual event. For extended and commendable achievement in dental materials research, the Wilmer Souder Award was presented by D. C. Hudson, Dental Materials Group president, to William T. Sweeney, of the National Bureau of Standards.

The Edward H. Hatton Award, having a monetary value of $100, was given to the novice research worker judged as having presented the most meritorious paper. There were 24 competitors in two separate sessions this year. At the banquet, E. B. Jump, chairman of the Hatton Award Committee, presented the certificate to Reginald J. Andlaw, of the Eastman Dental Dispensary, for his presentation entitled "The Relationship between Acid Production and Enamel Decalcification in Salivary Fermentations of Carbohydrate Food-stuffs." Honorable Mention was given to Hristo Chris Doku, of Tufts University School of Dental Medicine, for his paper "The Thromboplastic Activity of Human Saliva."

Conference on Research in Periodontal Disease.—Just preceding the IADR meeting an all-day conference on Research in Periodontal Disease was held by the Committee on Dentistry, National Academy of Sciences, National Research Council, on March 16 in Chicago, as a part of its regular annual meeting. The conference, held at the request of the Office of Naval Research, was designed to stimulate fundamental research in periodontal disease by suggesting new approaches for investigation or to point out wherein older approaches are in need of revision. Its aim was not so much to review previous findings as to explore the hypotheses underlying current research and to encourage speculation as to other ways in which basic science might be brought to bear on the stubborn problems of etiology and treatment.

In summarizing the proceedings, the conference chairman, Roy O. Greep, emphasized that while a host of factors have been implicated in periodontal disorders, there is still deep ignorance of their mechanisms of action and their relative importance as determinants of disease. Basic research in this field is disheartenigly limited, but the problems are susceptible to attack by a wide range of effective technics from histochemistry to epidemiology. It is hoped that the Proceedings, to be published as a supplement to the *Journal of Dental Research* with the support of the National Institutes of Health, will help plant the seeds of fundamental research in greater and more realistic proportions to the clinical importance of periodontal disease.

—F. J. O.

INTERNATIONAL ASSOCIATION FOR DENTAL RESEARCH ANNOUNCEMENT OF 1961 GENERAL MEETING

The 39th General Meeting of the International Association for Dental Research will convene at the Statler Hotel, Boston, Massachusetts, on Thursday, Friday, Saturday, and Sunday, March 23, 24, 25, and 26, 1961. The American Association of Dental Schools will meet at the same place, Sunday, March 26, to Wednesday, March 29, 1961.

DAN Y. BURRILL
Secretary-Treasurer

The Localization of Microörganisms in Inflamed Dental Pulps of Rats Following Bacteremia

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It has been recognized for some time that the dental pulp may be invaded by microorganisms either through pulpal exposures or by a vascular route. Robinson and Boling[1] studied the localization of streptococci and staphylococci in pulps of adult cats, some with naturally acquired infections and others with artificially induced bacteremias. In these over-all findings, pulps of 72 per cent of operated teeth and 8.5 per cent of control teeth contained bacteria when the animals were sacrificed. Robinson and Boling[1] were able to demonstrate that the severity of pulpal inflammation increased the chance of bacterial localization, but they were unable to correlate the age of the lesion with the localization of microörganisms. In comparison, Fox[2] and Rigdon,[3] working with tissues other than dental pulps, were able to show that the localization of bacteria from a hematogenous source occurred more frequently in severely inflamed localized lesions, than in less severel inflamed lesions in a similar location. In addition, younger lesions were more susceptible to localization than older ones.

The purpose of this investigation was to study the influence of irritation due to cavity preparation, filling materials, and medicaments on the localization and retention of blood-borne bacteria in pulpal tissues.

EXPERIMENTAL METHODS

An albino strain of Wistar rats, between the ages of 75 and 105 days, weighing approximately 150 gm., was selected for experimental study. Earlier reports by Maurice and Schour[4,5] and Weider, Schour, and Mohammed[6] and preliminary experiments in our laboratory have indicated that the first maxillary molars of rats are suitable for cavity preparations and studies on pulpal reactions.

A strain of *Staphylococcus aureus* was selected as a test organism because it demonstrated moderate virulence for the rat and was not fatal if injected intravenously in relatively large numbers. Furthermore, this organism was easy to isolate and identify by the use of a selective medium.*

Experimental animals were anesthetized by intraperitoneal injections of weight-graded doses of sodium nembutal† and were secured in a mouth prop.[7] Samples of saliva were streaked on the selective medium and examined later for the presence of *S. aureus*.

This study was supported by Research Grant D-338 from the N.I.D.R., U.S. Public Health Service.
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* Difco Staphylococcus Medium No. 110.

† Abbott Laboratories.

The maxillary teeth were isolated with small pledgets of cotton, dried, and the area cleansed with U.S.P. tincture of iodine. Cavities, with and without exposures, were prepared on the mesial aspect of first maxillary molars, using sterile ½ round and 33¼ inverted cone burs. Some of the operated teeth were treated with croton oil and then filled with zinc phosphate cement. The remaining cavities were restored with zinc phosphate cement or zinc oxide and eugenol.

Artificial bacteremias were induced by injecting into the femoral vein a 0.25-ml. amount of a suspension of the test organism grown in brain-heart infusion broth for approximately 18 hours at 37° C. It was estimated that approximately two hundred million organisms were placed in the blood stream with a single injection. Immediately following injection, a sample of tail blood was streaked on the selective medium, incubated at 37° C. and observed at intervals for growth of the test organism. Some animals were injected immediately after cavity preparations. In others a period of 24 hours intervened between cavity preparation and injection.

All animals were sacrificed on the first or seventh day following artificial bacteremia. This was accomplished with ether inhalation followed by decapitation. The maxillae were removed, washed in tap water, dried, and painted with U.S.P. tincture of iodine. The pulp chambers were opened with sterile ½ round burs.

TABLE 1*

PRESENCE OF *S. aureus* IN TOOTH PULPS FOLLOWING INTRAVENOUS INJECTION OF TEST ORGANISM

Interval between Injection and Sampling	Total	No. Positive	Percentage Positive
Less than 30 minutes	55	21	38
24 hours (heart blood positive)	153	18	12
24 hours (heart blood negative)	128	3	2

* Untreated (control) teeth.

Pulpal samples were obtained from treated and untreated control teeth from each animal. The exposed pulpal tissue was dissected free of the pulpal chamber with sterile explorers and picked up by sterile barbed broaches. The ends of broaches containing the pulpal samples were clipped with sterile scissors and allowed to fall into a tube containing brain-heart infusion broth. The tubes were incubated for 48 hours at 37° C. Samples from tubes showing growth of organisms were streaked on the selective medium and examined later for the presence of *S. aureus*.

One or more teeth selected at random from each group of animals was reserved for histopathologic study.

RESULTS

Data from pulps of untreated control teeth appear in Table 1. Samples from 55 pulps obtained within 30 minutes after injection of the test organism indicated that 21, or 38 per cent, of these pulpal samples were positive when tested for the presence of *S. aureus*. In animals whose blood was positive for the test organism 24 hours after injection 18, or 12 per cent, of pulps from 153 teeth were positive, whereas from animals whose blood stream was free of bacteria after 24 hours only 3, or 2 per cent,

TABLE 2*

GROUP A: PRESENCE OF *S. aureus* IN TOOTH PULPS FOLLOWING INTRAVENOUS INJECTION OF TEST ORGANISM

FILLING MATERIALS	EXPOSURES			NON-EXPOSURES		
	Total No.	No. Positive	Per Cent Positive	Total No.	No. Positive	Per Cent Positive
Croton oil and cement	49	27	55	43	13	30
Cement	39	25	64	16	6	38
Eugenol and zinc oxide	38	30	79	16	6	38

* Cavity preparation to injection, within 30 minutes; injection to sampling, 24 hours.

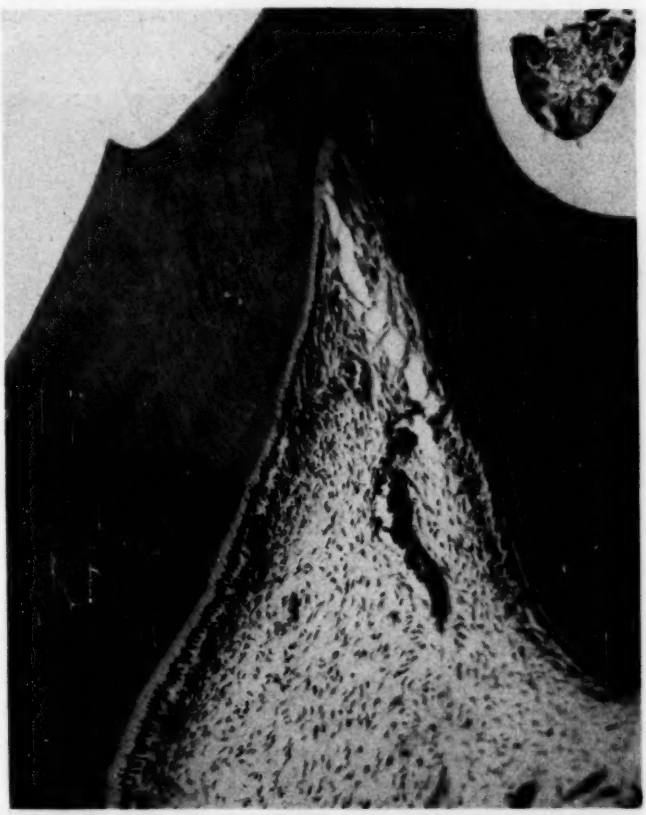

FIG. 1.—Non-exposure, restored with zinc oxide and eugenol, 1 day postoperative. Note the very mild inflammatory reaction throughout the pulp. The odontoblastic layer is intact except for a small area of cell loss at the coronal tip of pulp tissue. (Hematoxylin and eosin; orig. mag. ×100.)

of pulps from 128 teeth were positive. Colony counts from heart blood samples usually contained from 10 to 100 bacteria per milliliter in the series listed as heart blood—positive. In exceptional cases massive bacteremias were noted at 24 hours or later, and such animals were not included in data recorded in this report.

Data from group A animals are presented in Table 2. These rats were injected with the test organism within 30 minutes following operative procedures and sacrificed 24 hours later. There was a great difference between the 55–79 per cent of positive cultures obtained from exposed pulps and the 30–38 per cent of positive cultures obtained from non-exposed pulps. Histopathologic evidence indicated that the exposed pulps were much more severely inflamed than non-exposed pulps, as noted in Figures 1–5.

Data from group B animals appear in Table 3. The test organism was injected 30 minutes after cavity preparation, and pulpal samples were obtained 7 days later. Positive cultures were obtained from 66 per cent of the croton-oil–treated exposed pulps, as compared with 21.4–27.5 per cent of the remaining exposed pulps. Cultures from non-exposed pulps were uniformly negative.

FIG. 2.—Non-exposure, restored with zinc oxide and eugenol, 7 days postoperative. Mild inflammatory reaction. Extravascular hemorrhage was likely produced mechanically at the time of sacrifice and is not related to the lesion. (Hematoxylin and eosin; orig. mag. ×100.)

FIG. 3.—Pulp exposure, capped with zinc oxide and eugenol, 1 day postoperative. Some dentin chips have been pushed through the aperture of the exposure site. Pulpal tissue immediately adjacent shows evidence of necrotic change with loss of cell clarity and pyknosis of remaining nuclei. Deeper vessels of pulp are engorged. (Hematoxylin and eosin; orig. mag. ×100.)

TABLE 3*

GROUP B: PRESENCE OF *S. aureus* IN TOOTH PULPS FOLLOWING INTRAVENOUS INJECTION OF TEST ORGANISM

FILLING MATERIALS	EXPOSURES			NON-EXPOSURES		
	Total No.	No. Positive	Per Cent Positive	Total No.	No. Positive	Per Cent Positive
Croton oil and cement	41	27	66	25	0	0
Cement	42	9	21.4	4	0	0
Eugenol and zinc oxide	40	11	27.5	8	0	0

* Cavity preparation to injection, within 30 minutes; injection to sampling, 7 days.

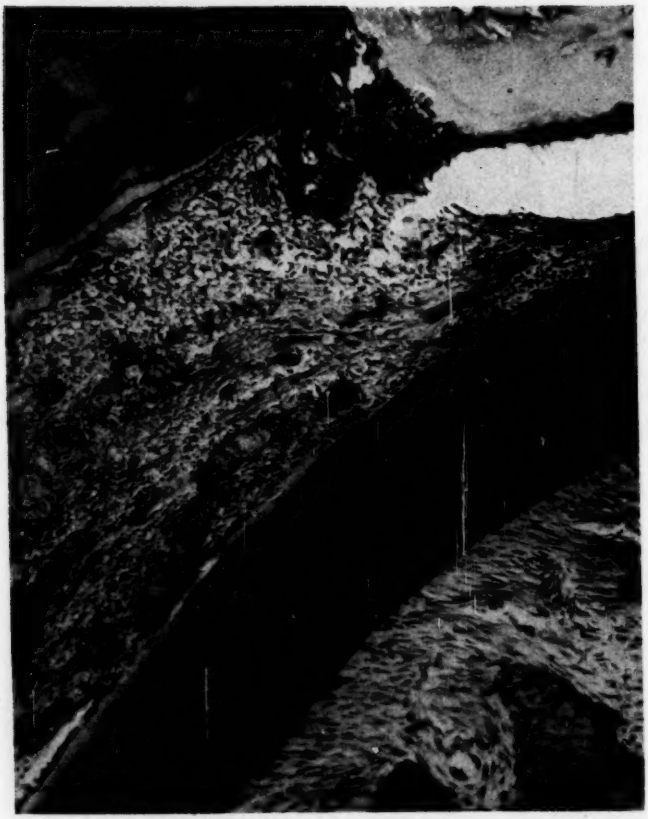

FIG. 4.—Pulp exposure, capped with zinc oxide and eugenol, 7 days postoperative. Note the accumulation of a large number of clear vacuolated cells and an adjacent developing pyogenic membrane. Pulpal vessels are markedly engorged. (Hematoxylin and eosin; orig. mag. ×100.)

TABLE 4*

GROUP C: PRESENCE OF *S. aureus* IN TOOTH PULPS FOLLOWING INTRAVENOUS INJECTION OF TEST ORGANISM

FILLING MATERIALS	EXPOSURES			NON-EXPOSURES		
	Total No.	No. Positive	Per Cent Positive	Total No.	No. Positive	Per Cent Positive
Croton oil and cement	39	19	49	23	0	0
Cement	39	14	36	10	0	0
Eugenol and zinc oxide	39	7	18	0	0	0

* Cavity preparation to injection, 24 hours; injection to sampling, 24 hours.

Data from group C animals appear in Table 4. The test organism was injected 24 hours after cavity preparation, and pulpal samples were obtained 24 hours later. The pattern of experimental data in this group is quite similar to that outlined in Table 3, with a continued high incidence of 49 per cent positive pulps from croton-oil–treated exposures, whereas positive pulps were detected in 18–36 per cent of exposed pulps treated with zinc oxide and eugenol or zinc phosphate cement. Non-exposed pulps from this series were uniformly negative.

Data for group D animals appear in Table 5. The test organism was injected 24 hours after cavity preparation, and pulpal samples were obtained 7 days later. The test organism was recovered in only 2.6 per cent of the croton-oil–treated exposures and 4.2 per cent of the zinc phosphate cement–treated exposures. The zinc oxide and eugenol-treated exposures were uniformly negative when tested for the presence of *S. aureus*. Similar treatment of non-exposed pulp was not studied in this series.

Colonies resembling the test organism were found in less than 10 per cent of saliva samples. However, as a further control against false positive cultures, pulps of 20 first

FIG. 5.—Pulp exposure, treated with croton oil and capped with zinc phosphate cement, 7 days postoperative. Note the widened necrotic zone adjacent to the aperture of the exposure. No pyogenic membrane is evident. (Giemsa stain; orig. mag. × 100.)

molars, in 10 animals, were mechanically exposed and filled with zinc phosphate cement. No artificial bacteremia was established in these animals, and pulpal samples obtained 24 hours later were consistently negative when tested for the presence of organisms similar to the test organism.

DISCUSSION

The differences between non-exposed pulps and exposed pulps in all series of experimental conditions presented in this study are quite obvious and require no additional statistical analysis. This is substantiated by the fact that all non-exposed pulps except those of group A were negative when tested for the presence of *S. aureus*. Thus the incidence of bacterial localization was greater in pulps that were more severely inflamed by mechanical exposure, and the bacteria were retained in the pulpal tissues for a longer period of time. Figures 3, 4, and 5 indicate that pulpal exposure produces a more severely inflamed lesion, as compared with the milder reaction observed under non-exposed cavity preparations in Figures 1 and 2. This observation is in agreement with that of Robinson and Boling,[1] Fox,[2] and Rigdon.[3]

TABLE 5*

GROUP D: PRESENCE OF *S. aureus* IN TOOTH PULPS FOLLOWING INTRAVENOUS INJECTION OF TEST ORGANISM

FILLING MATERIALS	EXPOSURES			NON-EXPOSURES
	Total No	No Positive	Per Cent Positive	
Croton oil and cement	39	1	2.6	Not done
Cement	48	2	4.2	
Eugenol and zinc oxide	39	0	0	

* Cavity preparation to injection, 24 hours; injection to sampling, 7 days.

TABLE 6

SUMMARY OF ANALYSIS OF VARIANCE*

Source	Chi Square	Source	Chi Square
Group A versus group B	21.0	Groups AB versus groups CD	93.4
Group C versus group D	60.7	Croton oil and cement versus zinc oxide–eugenol and cement	8.2
Group A versus group C	27.3	Cement versus zinc oxide–eugenol	0.6
Group B versus group D	71.2		

* $P > 0.05$ when the chi square is 3.84 or greater.

The data from exposed pulps of the various groups of animals were compared by utilizing the analysis of variance. The results of these comparisons appear in Table 6. In order to obtain a degree of significance to the level of 0.05, the chi square must equal or exceed 3.84.

A comparison of all exposures from group A with those of group B yielded a chi square of 21.0. Thus the differences are significant. It is to be noted, however, that the

croton-oil–treated exposures in both groups followed a similar pattern. Therefore, any differences that achieve a level of significance must be due mainly to the lowered incidence of positive pulp findings observed in the zinc phosphate cement– and zinc oxide and eugenol–treated exposures of group B. Both groups of animals were injected with bacteria immediately following cavity preparation, but the group B animals were given a longer period of time to allow for the operation of whatever bodily defense mechanisms were mobilized to clear organisms that probably localized in these pulps in approximately the same percentages as those indicated in the group A exposures.

The differences between all exposures in group C as compared to group D measured well within the limits of significance. This analysis, as in the groups AB comparison, yields information on the ability of the group D animals to clear the dental pulp of organisms, since the pulpal samples were obtained 1 week later than those of group C. Both groups of animals were injected with the test organism 1 day after cavity preparation. As noted in the former analysis, croton-oil–treated animals continued to demonstrate a relatively high percentage of positive pulp findings when sampled 1 day after bacteremia, with zinc phosphate cement–treated animals next highest and zinc oxide and eugenol–treated animals the lowest. The dental pulps under all three filling materials or medicaments behaved somewhat similarly when sampled 1 week later, showing a significant reduction in the number of instances that the test organisms appeared in the pulpal tissues.

In order to test for any significant reduction in the incidence of localization and retention of organisms in older lesions as compared with recently developed lesions, comparisons were made between groups A versus C and groups B versus D. Since both these comparisons measured to a significant level, it can be stated that, when bacteremia occurred at least 1 day following initiation of an inflammatory lesion, there was less tendency for localization to occur and those organisms that did localize tended to be cleared in a larger number of instances after 1 week. This time relationship is evident in the work of Fox[2] and Rigdon.[3] The work of Robinson and Boling[1] did not measure any differences in the incidence of bacterial localization in young or older lesions of the dental pulps of cats.

The effect upon the exposed pulp under zinc phosphate cement or zinc oxide and eugenol seemed to make little difference in the over-all comparison, in spite of the fact that there was some minor variation within a single group, since the chi square in this comparison is 0.6. The use of croton oil, however, seemed to delay the clearing action of bacteria localized in younger lesions and increased the incidence of localization in older lesions. The chi square is significant when a comparison is made between exposures treated with croton oil and those not treated with this medicament. Histopathologic evidence of the condition of dental pulps after 7 days following pre-treatment with croton oil, as seen in Figure 5, indicates that these pulps were more severely inflamed and showed fewer localized lesions. There was a more extensive cellular infiltration, with no pyogenic membrane in the area between inflamed tissues and those tissues less affected.

In this study the relatively small percentage of bacterial localization in pulps of non-exposure–treated teeth casts doubt upon the value of the rat as a suitable animal for testing the merits of medicaments. On the other hand, it is possible that rats may be useful in comparing the local antibacterial action of medicaments over exposures.

SUMMARY

The pulps of 993 rat molars were analyzed for bacterial content following intravenous injection of *S. aureus*. Exposed and non-exposed pulps were studied with a variety of filling materials and variations in the time of sacrifice and injection of the test organism. Bacterial localization occurred more frequently among exposed pulps. When the time of injection or the date of sacrifice was delayed, the incidence of bacterial localization was reduced in all series except croton-oil–treated exposures. This study is accompanied by a statistical and histopathologic analysis.

The authors wish to acknowledge the assistance of Dr. Harold Blank, who prepared the statistical analysis.

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Periodontal Disease in Six Strains of Inbred Mice

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Recent investigations have shown that certain strains of inbred mice are susceptible to periodontal disease.[1, 2] The aim of the present investigation was to determine whether additional susceptible strains could be found and to test a method of presenting data which would establish the relative susceptibility of the different strains to periodontal disease.

MATERIALS AND METHODS

The maxillae and mandibles of 246 inbred mice, 106 males and 140 females, 12 to 24 months of age, of the strains SWR/N, Bl/HeN, BRSUNT/N, C57Bl/6JN, A/HeN, and A/LN were examined (see Table 1). The mice were 30 days of age at the

TABLE 1

NUMBER OF ANIMALS

Strain	Male	Female	Total
A/LN	22	27	49
A/HeN	15	29	44
C57Bl/6JN	21	21	42
BRSUNT/N	17	24	41
Bl/HeN	18	21	39
SWR/N	13	18	31
Total	106	140	246

start of the experimental period. They were housed in plastic cages in groups of 4 to 7 and were maintained on Purina Laboratory Chow and water ad libitum. At the time of sacrifice the mice were killed with ether, and the heads were removed and fixed in 10 per cent formalin. The heads of the animals were then split in a mid-saggital plane. Half of all the heads were then defleshed by the papain method, and, in addition, six specimens from each strain of the remaining half-heads were selected for histologic examination. The latter were decalcified and sectioned serially at 6μ. Three of these six specimens were sectioned in a mesiodistal direction and three in a buccal-lingual direction. The sections were stained with hematoxylin and eosin.

The defleshed specimens were scored for alveolar bone loss according to the method previously described.[2] In brief, this consisted of scoring each molar tooth from 0, indicating little or no bone loss, to 4, indicating severe involvement. For purposes of statis-

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tical analysis the animals in all the age groups were pooled, as it was apparent from the raw data that little or no difference existed in the periodontal scores after 12 months of age. The scores of the alveolar bone loss were then transformed to ridits and analyzed.[3] Ridits can be best described in Bross's own words: "The name 'ridits' was chosen because of the analogy with 'probits' and 'logits.' Like other members of the 'it' family ridits represent a type of transformation. But whereas probits are relative to a theoretical distribution (the normal distribution), ridits are relative to an empirical distribution. The first three letters stand for Relative to an Identified Distribution."

The identified distribution used here was the distribution of the total scores of the mandibular molars for strain A/LN. Since the possible values of single-tooth periodontal scores ranged from 0 through 4, the possible range of the combined scores for the three molar teeth was 0 through 12. Total scores of 0 through 2 (representing

TABLE 2

DISTRIBUTION OF ANIMALS WITHIN STRAIN BY MANDIBULAR SCORE

Score	Strain					
	A/LN	A/HeN	C57Bl/6JN	BRSUNT/N	Bl/HeN	SWR/N
0–2	4	.....	.....	.....	.....	4
3	3	.....	1	.....	.....	1
4	3	.....	0	.....	.....	1
5	10	1	7	.....	.....	0
6	7	6	2	1	1	5
7	7	3	3	1	0	3
8	6	14	4	0	4	0
9	5	9	6	2	10	4
10–12	4	11	19	37	24	13
Total	49	44	42	41	39	31

slight periodontal involvement) and 10 through 12 (indicating most severe periodontal disease) were arbitrarily pooled, in order to smooth the distribution, as these extreme scores were more susceptible to error of classification. The periodontal scores for the mandibular and maxillary arches for the other strains were treated in a similar manner. A study of Table 2 and 3 reveals that the mandibular arch only of animals of strain A/LN was represented in every scoring category from 0 to 12. This was the main reason for selecting the mandibular arch of this strain to provide the identified distribution.

Table 4 illustrates the procedure for calculating the ridits. All raw scores were translated into ridits and averaged by strain and sex. A requirement of the identified distribution obtained from the reference strain is that it must have an average ridit of 0.5, so that an animal picked at random from the reference strain will have an equal chance of having a higher or lower periodontal score than the score of another animal picked at random from the reference strain. This explanation also applies to comparisons of the reference strain with other strains. Thus the average ridit of 0.750 for strain C57Bl/6JN should be interpreted as the probability that a randomly selected animal

from strain C57Bl/6JN would have a higher periodontal score than an animal selected at random from the reference strain.

A simple method for testing whether the differences noted in the tables are statistically significant is to assign confidence limits (at the level of probability desired) to average ridits (see Table 5); all those confidence limits which encompass the value 0.5 are statistically not significant from the identified distribution at the probability level at which the confidence limit is computed. To set up the confidence limits, the variances of the ridits were calculated (Table 6). These variances were then converted to the appropriate standard errors, multiplied by the appropriate *t*-value, and added to, or substracted from, the mean ridit, to give, in this case, 95 per cent confidence limits (Table 7).

TABLE 3

DISTRIBUTION OF ANIMALS WITHIN STRAIN BY MAXILLARY TOTAL SCORE

Score	Strain					
	A/LN	A/HeN	C57Bl/6JN	BRSUNT/N	Bl/HeN	SWR/N
0–2	.....	.....	1	.....	.....	2
3	.....	.....	1	.....	.....	1
4	.....	.....	3	.....	.....	4
5	.....	.....	2	1	.....	1
6	3	1	5	0	3	2
7	2	2	2	2	3	0
8	1	2	1	0	2	1
9	0	0	0	2	0	0
10–12	43	39	27	36	31	20
Total	49	44	42	41	39	31

TABLE 4*

CALCULATION OF RIDITS BASED ON IDENTIFIED DISTRIBUTION OF TOTAL MANDIBULAR SCORES OF STRAIN A/LN

Mandibular Scores	1	2	3	4	5
0–2	4	2.0	0	2.0	0.041
3	3	1.5	4	5.5	.112
4	3	1.5	7	8.5	.173
5	10	5.0	10	15.0	.306
6	7	3.5	20	23.5	.480
7	7	3.5	27	30.5	.622
8	6	3.0	34	37.0	.755
9	5	2.5	40	42.5	.867
10–12	4	2.0	45	47.0	0.959
Total	49	.....	49	.....	.....

* The columns as numbered have the following meanings:
1. The frequency distribution in the identified distribution (reference class).
2. Half the corresponding entry in col. 1.
3. The cumulative of col. 1, displaced one category downward.
4. Column 2+col. 3.
5. The ridits which are calculated as the entries in col. 4 divided by the total (49).

RESULTS

Gross and histologic observations.—Figures 1 through 4 illustrate typical gross and histologic observations made on strains A/LN and A/HeN. These two strains had severe hair impaction (*HI*), which tended to be confined to the lingual surfaces of the maxillary molar teeth, and the hairs were actually located between the tooth and the palatal plate of cortical bone (*PB*). The buccal plate (*BP*) of bone was seen to be

TABLE 5

AVERAGE RIDITS BY STRAIN AND SEX

Sex	Strain					
	A/LN	A/HeN	C57Bl/6JN	BRSUNT/N	Bl/HeN	SWR/N
	Mandible					
Male	.....	0.771	0.600	0.906	0.883	0.585
Female	.....	0.781	0.901	0.955	0.919	0.702
Total	.....	0.778	0.750	0.935	0.902	0.657
	Maxilla					
Male	0.891	0.891	0.567	0.876	0.830	0.664
Female	0.929	0.935	0.936	0.955	0.933	0.743
Total	0.912	0.920	0.752	0.922	0.886	0.712

TABLE 6

VARIANCES OF RIDIT SCORE, BY STRAIN AND SEX

Sex	Strain					
	A/LN	A/HeN	C57Bl/6JN	BRSUNT/N	Bl/HeN	SWR/N
	Mandible					
Male	0.0856	0.0416	0.0759	0.0189	0.0152	0.1305
Female	0.0748	0.0239	0.0215	0.0004	0.0033	0.1164
Total	0.0796	0.0305	0.0487	0.0080	0.0088	0.1217
	Maxilla					
Male	0.0244	0.0225	0.1116	0.0340	0.0388	0.1630
Female	0.0122	0.0091	0.0109	0.0004	0.0071	0.1155
Total	0.0177	0.0136	0.0612	0.0141	0.0216	0.1335

TABLE 7

UPPER AND LOWER CONFIDENCE LIMITS (95 PER CENT) ON AVERAGE RIDITS IN MALES AND FEMALES OF ALL STRAINS

SEX	STRAIN					
	A/LN	A/HeN	C57Bl/6JN	BRSUNT/N	Bl/HeN	SWR/N
	Mandible					
Male:						
Upper		0.635	0.446	0.819	0.809	0.319
Lower		.907	.754	.993	.957	.851
Female:						
Upper		.907	.819	.945	.887	.502
Lower		0.855	0.983	0.965	0.951	0.902
Total:						
Upper		0.711	0.663	0.899	0.864	0.496
Lower		0.845	0.837	0.971	0.940	0.818
	Maxilla					
Male:						
Upper	0.807	0.791	0.380	0.763	0.712	0.367
Lower	.975	.991	.754	.989	.948	.961
Female:						
Upper	.875	.889	.877	.945	.887	.543
Lower	0.983	0.981	0.995	0.965	0.979	0.943
Total:						
Upper	0.863	0.874	0.655	0.876	0.827	0.543
Lower	0.961	0.966	0.849	0.968	0.945	0.881

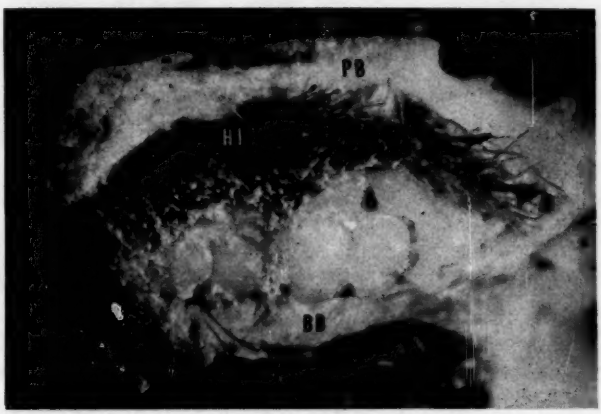


FIG. 1.—An example of severe hair impaction which occurred on the palatal surfaces of the maxillary molars in strains A/LN and A/HeN. *PB* = palatal bone; *HI* = hair impaction; *BB* = buccal bone.

FIG. 2.—Hair was carefully removed to demonstrate the amount of alveolar bone loss associated with the hair impaction.

FIG. 3.—Mesiodistal section through maxillary molars to demonstrate the depth to which hair can penetrate. *HI* = hair impaction; *R* = root; *B* = alevolar bone.

intact and relatively free of hair impaction. When the impacted hairs were carefully removed from about the lingual surfaces of the teeth (Fig. 2), almost the entire length of root became exposed to view. The supporting alveolar bone had become cupped or hollowed out, and only thin buccal and lingual plates of cortical bone remained.

The impacted hairs (*HI*) penetrated to the apices of the roots (*R*) and even beyond, and the interproximal supporting tissues were almost completely destroyed (Fig. 3). Only a thin plate of cortical bone (*B*) appears to be left.

In contrast to the maxillary molars, the mandibular molars (Fig. 4) from the same animal as that shown in Figure 1 revealed an absence of impacted hairs. Although some alveolar bone loss occurred, particularly about the third molar and the mesial root of the first molar, the bone loss was a horizontal one and not the hollowing-out type of destructive lesion observed with hair impaction (Fig. 2).

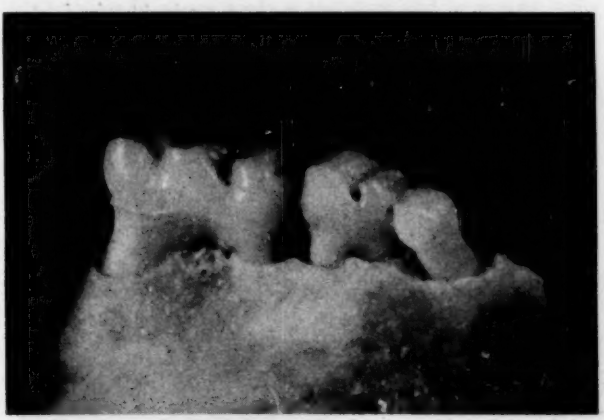


FIG. 4.—Mandibular molars of animal shown in Fig. 1. The absence of hair impaction in the mandibular arch is striking.

In strains SWR/N, Bl/HeN, and C57Bl/6JN hair impaction also occurred, but to a much more limited extent, and appeared to have caused an intensification of alveolar bone loss in the localized areas where it was found (Fig. 5, for example).

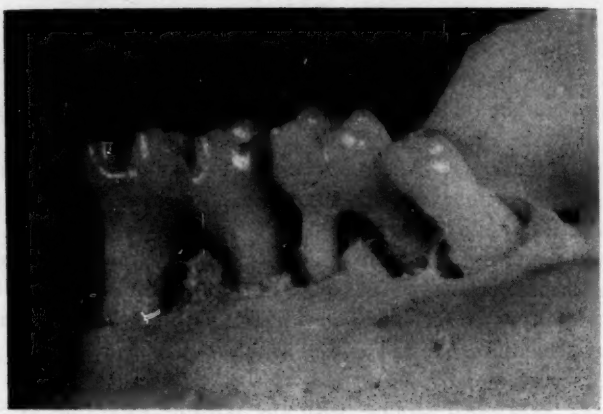
Strain BRSUNT/N was the only strain in this experiment which had severe loss of alveolar bone around the molar teeth in both jaws and in males as well as females, but with little or no hair impaction or retention present (Fig. 6). The severe loss of supporting alveolar bone which occurred was a horizontal one. Again, this was in marked contrast to the hollowing-out of the alveolar bone observed in strains A/LN and A/HeN, which had hair impaction.

The epithelial attachment (*EA*) in strain BRSUNT/N migrated apically and was located at approximately half the root length (Fig. 7). This resulted in the formation of a true periodontal pocket (*P*) lined on one side with crevicular epithelium and on the other side by cementum. Crestal, as well as interradicular, resorption of supporting bone was also observed.

Analysis of the data.—All strains, with the possible exception of SWR/N, had a greater probability of higher mandibular periodontal scores than did the reference

FIG. 5.—This limited amount of hair impaction is characteristic of strains C57Bl/6JN, SWR/N, and Bl/HeN.

A



B

FIG. 6.—Severe loss of alveolar bone in the absence of hair impaction is seen in strain BRSUNT/N. *A* = maxillary arch; *B* = mandibular arch.

strain A/LN. The probabilities ranged from 0.935 for strain BRSUNT/N to 0.657 for strain SWR/N (Table 5). The latter probability, however, had 95 per cent confidence limits of 0.496 and 0.818 (Table 7), a range which includes the value 0.5. It is possible that, because of sampling error, the estimated average value (0.657) did not reflect the true average value, which might have been nearer 0.5. The females in all strains had more severe periodontal involvement in the mandibles than did the reference strain, but the males did not (Tables 5 and 7). The average ridits for female animals in all strains ranged from 0.702 for strain SWR/N to 0.955 for BRSUNT/N; statistically, all these scores were significantly higher than 0.5 (Table 7). The ridits

FIG. 7.—Bu-Li section through maxillary molar tooth of strain BRSUNT/N to show formation of a true periodontal pocket. *EA* = epithelial attachment; *P* = periodontal pocket; *B* = alveolar bone.

of males of strains SWR/N and C57Bl/6JN, on the other hand, did not differ significantly from those of the reference strain.

All strains had more severe periodontal involvement in the maxillary arch (Tables 5 and 7) than the mandibular arch of the reference strain. The females of all strains had more severe involvement than the males. The average ridits for the females in all strains were larger than 0.5. These differences were statistically significant (see Table 7 for confidence limits). The males of strains SWR/N and C57Bl/6JN, however, did not have ridits which were significantly different.

Strain C57Bl/6JN showed the most significant sex difference in regard to loss of alveolar bone. The females had more loss than the males. Strains A/LN and A/HeN showed the most marked difference in periodontal disease (alveolar bone loss) between the two arches. The greatest destruction was on the maxillary arch. The greatest amount of over-all loss of alveolar bone, however, occurred in strain BRSUNT/N.

DISCUSSION

Strains A/LN and A/HeN, which had gross hair impaction occurring on the palatal aspect of the maxillary molar teeth, are known to suffer from alopecia. These animals cleanse themselves with their tongues. This behavior undoubtedly is responsible for large quantities of loose hair being lodged upon them. During the act of swallowing, the tongue is forced upward and backward, and this, in all likelihood, is responsible for the hairs being forced into the crevices between the teeth and the alveolar bone. Neither grossly nor histologically, therefore, did the periodontal lesions found in the areas of hair impaction in these two strains resemble the chronic type of lesion found in the human.

The difference in the amount of alveolar bone loss between the sexes in strain C57Bl/6JN is difficult to explain, although such differences are not unusual in animal work. Similar differences, for instance, have been reported in the hamster by Costich[4] and Gold and Keyes.[5]

Strains SWR/N, Bl/HeN, and C57Bl/6JN also had hair impaction, but to a much more limited extent than that found in strains A/LN and A/HeN. It should be pointed out, however, that there were always some areas in the five above-mentioned strains that were free of hair impaction. In these areas the histologic picture was naturally quite different from that described for the areas in which hair impaction did occur. That is, when no bone loss had occurred, the epithelial attachment was found at the cementoenamel junction. On the other hand, where some horizontal loss of alveolar bone had occurred, a true periodontal pocket was found.

It was only in strain BRSUNT/N that a chronic type of lesion with true pocket formation, similar to that seen in the human, was universally found. It is apparent, therefore, that, of all six strains, this one is probably the most suitable for use in periodontal research.

SUMMARY AND CONCLUSIONS

1. The scores of the mandibular molar teeth of strain A/LN, selected as the reference strain or identified distribution, were statistically analyzed by means of the ridit method.

2. The females of all strains except the reference strain had more severe periodontal

disease in both arches than did the reference strain. This difference was statistically significant.

3. The maxillary arch of all strains showed more severe periodontal disease than did the mandibular molars of the reference strain A/LN.

4. Strains A/LN and A/HeN had severe hair impaction on the palatal surface of the maxillary molars and severe loss of alveolar bone in the maxillary arch as compared with the mandibular arch.

5. Hair impaction resulted in almost complete destruction of the supporting spongy bone around the molar teeth. Observed grossly, this appeared as a hollowing-out of the bone immediately adjacent to the teeth, with exposure of the roots down to their apical ends.

6. Strain C57Bl/6JN showed the most marked sex difference in regard to alveolar bone loss, the females having the greater loss.

7. Strain BRSUNT/N had the greatest loss of alveolar bone in both arches and in both sexes. In addition, histologic observation revealed that this strain developed a true periodontal pocket. Strain BRSUNT/N appeared to be the most suitable for use in periodontal research.

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Cytotoxic Effect of Dental Local Anesthetics on Tissue Culture

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The frequent use of local anesthetics in dental procedures and the clinical reactions they sometimes induce emphasize the need for a study of their possible cytotoxic effect. Bjorlin[1] demonstrated that tissue injury occurred in mice following injection of lidocaine and procaine. He observed degenerative changes and necrosis of the connective tissue plus vascular changes, including ruptured venules and capillaries, and necrosis of vessel walls. However, in vivo investigations consume much time and require the use of animals, animal quarters, caretakers, and special experimental conditions, and the results are often equivocal and difficult to evaluate because of individual differences in animals. Many of these objections may be obviated by the use of tissue cultures as the basal test material. The investigator has a closer control of experimental conditions, and the effect on individual cells or tissues can be evaluated better.

Utilizing tissue-culture techniques, Pomerat[2] tested many substances, including epinephrine and procaine, for their effects on chick embryonic and human tissue explants and found that procaine HCl, at a concentration of 3–6 μg/ml of medium was injurious to chick heart cells. Koch, Goldberg, and Gralnick[3] investigated various local anesthetic solutions as to tissue reactions. They questioned the compatibility of such solutions with other living cells and tissues.

The use of tissue-culture techniques already has widespread applications in a variety of medical research fields. In the present study an attempt was made to adapt these techniques to estimate the comparative cytotoxic effects of various commercial preparations of local dental anesthetics in common use today.

MATERIALS AND METHODS

A. *Anesthetics.*—These were commercial preparations of commonly used dental local anesthetics. The preparations are numbered 1 through 7; the seventh preparation, however, consists of three forms of the same anesthetic, each preparation differing from the other two by its content of epinephrine: 7a = anesthetic + epinephrine 1–100,000; 7b = anesthetic + epinephrine 1–50,000; 7c = anesthetic without epinephrine. Preparation 8 is standard procaine 2 per cent, and 9 is a control using Hanks' Balanced Salt Solution (BSS) made up to pH 4.0, which is well within the range of acidity of the anesthetic preparations used.

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B. *Test cell cultures.*—Test-tube monolayer cultures of monkey kidney and HeLa cells were used. These cultures were obtained from Microbiological Associates, Bethesda, Maryland.

C. *Media.*—Morgan, Morton, and Parker's[4] mixture No. 199 + 10 per cent horse serum was used as the nutrient medium for the monkey kidney cells; Eagle's basal medium[5] + 10 per cent horse serum was used for the HeLa cells.

D. *Stain.*—An aqueous solution of trypan blue, 0.5 per cent, was used.

PROCEDURE

Test-tube monolayer cultures of monkey kidney and HeLa cells were examined microscopically, and only those which showed good confluent growth were used. The culture media was decanted, and the cells were washed twice with Hanks' BSS. One milliliter of each anesthetic solution was added to separate cell culture tubes and allowed to remain in contact with the cells for varying time intervals. At the end of each interval the anesthetic was removed, and the cell culture was washed several times to remove all traces of the anesthetic. One milliliter of 0.5 per cent aqueous trypan blue was added and permitted to remain in contact with the cells for 15–30 seconds. The stain was then removed and the excess washed off gently with either Hanks' BSS or saline. The tubes were examined microscopically to estimate the percentage of viable (colorless) and non-viable (blue) cells. In order to check viability and non-viability, duplicate cell cultures were incubated with fresh nutrient after contact with the anesthetics. A drop in pH in the media within 3 days indicated viability; an unchanged pH indicated non-viability. As a control, Hanks' BSS, adjusted to pH 4.0, was used; this was the lowest pH of any of the anesthetics.

Attempts were made to do an actual direct count of the viable (colorless) and non-viable (blue) cells in a hemocytometer. Success was achieved with the HeLa culture. One milliliter of 0.1 M citric acid was added to the stained culture tubes, and the cells were scraped off with a rubber policeman. The suspension was gently mixed with a capillary pipette, and 1 drop was added to the counting chamber. A direct count of stained and unstained nuclei was made. This method, however, could not be used with the monkey kidney cells because too much cellular debris was found when the cells were scraped from the sides of the tubes.

RESULTS

Comparative cytotoxic effects of the various anesthetics are indicated in Table 1. These results are based on observations of viability as described under "Procedure." In all cases duplicates of tubes in which all the cells were stained and in which the test agents were removed and replaced by fresh nutrient failed to metabolize, as was indicated by lack of change in pH even after 3–4 days. Duplicates of tubes which contained colorless cells always produced a drop in pH. There was always a close agreement between the direct count and the estimate of viable and non-viable cells.

Our investigations seem to indicate that the effects of the anesthetics are parallel for the monkey kidney and HeLa cells. Most of the anesthetics show little cytotoxicity at the end of 15 minutes. The differences in the effects of the various preparations become emphasized after 30 minutes of exposure, most cytotoxicity being noted with

anesthetic No. 3. With this anesthetic, slight cytotoxicity is observed even after 15 minutes, increases rapidly, and almost complete destruction occurs in 30 minutes.

Anesthetic No. 6 ranks second in cytotoxicity; considerable cellular destruction is apparent after 30 minutes, and at the end of 1 hour most of the cells are destroyed. The remaining anesthetics showed little differences, although anesthetic 7c (anesthetic No. 7 without epinephrine) was the most cytotoxic. The anesthetic No. 7 combinations with epinephrine were less cytotoxic than the same anesthetic without epinephrine. Least cytotoxicity was observed with anesthetics Nos. 1 and 2. These compounds compared favorably with 2 per cent procaine, which is often used as a standard. After 3 hours of contact, all the anesthetics were completely cytotoxic.

TABLE 1*

CYTOTOXIC EFFECT OF DENTAL LOCAL ANESTHETICS ON MONKEY KIDNEY AND HeLa CELLS

Anesthetic No.	pH	Contact Time (Hours)							
		¼		½		1		2	
		MK	HeLa	MK	HeLa	MK	HeLa	MK	HeLa
1	4.3	0	0	5	10	15	20	70	100
2	4.5	0	5	0	5	15	20	70	80
3	4.2	20	20	50	80	80	100	100	100
4	4.4	0	0	5	5	15	30	100	100
5	4.4	5	0	10	5	15	30	100	100
6	4.5	10	5	35	20	50	80	100	100
7a	4.8	0	10	15	10	30	50	80	100
7b	4.7	0	5	15	10	30	50	80	100
7c	6.8	0	5	15	10	50	70	100	100
8	4.1	0	0	0	10	10	20	80	100
9	4.0	0	0	0	0	0	0	0	0

* Figures represent percentage destruction of cells.

DISCUSSION

The use of cell-culture techniques is expanding rapidly. In virology they are used for the cultivation of viruses, as well as for the assay of viral antibodies. They are used in cancer studies also. In pharmacology, cell-culture techniques are finding their place in investigation of drug action. However, the techniques do have their drawbacks; as yet they do not permit accurate quantitative determinations, as was soon apparent during the course of the present investigation.

Early in the study, cytotoxic activity was estimated from 0 to 4+, based on granulation and vacuolization of the cells, according to the technic of Robbins, Enders, and Weller.[6] This method proved to be inadequate under the present experimental conditions. In many cases cells that were later found to be non-viable had the same apperance microscopically as did control cells. However, when fresh nutrient was added, these cells failed to metabolize, as indicated by lack of change of pH, even after several days' incubation. Many anesthetics were actually fixing the cells to the glass without causing visible morphologic change (cf. Figs. 1 and 2). Figure 1 rep-

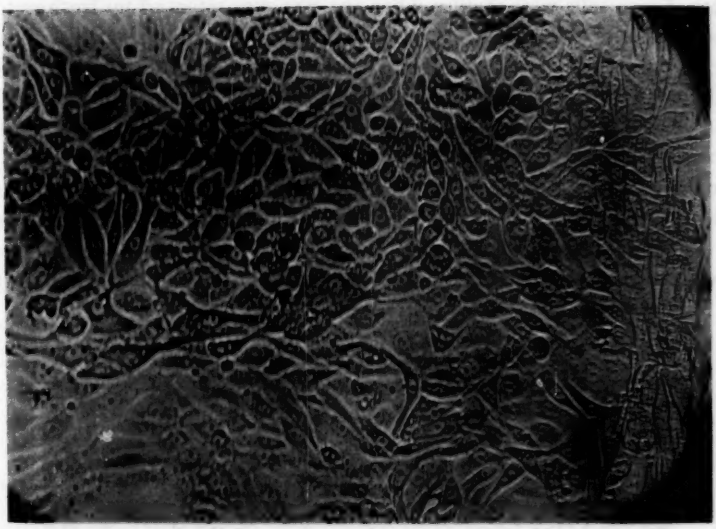

FIG. 1.—Control, monkey kidney (MK) cells exposed to Hanks' BSS, pH 4.0, for 30 minutes. Unstained.

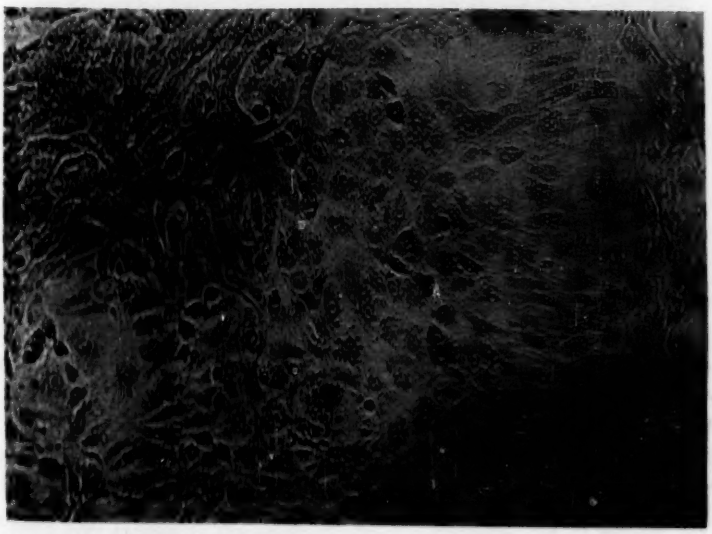

FIG. 2.—MK cells exposed to the anesthetic No. 3 for 30 minutes. Unstained

resents a control culture of monkey kidney cells exposed to Hanks' BSS, pH 4.0, for 30 minutes. These cells were viable; when the BSS was replaced by fresh nutrient, the cells showed definite evidence of metabolic activity by a fall in pH in 1–2 days. Figure 2 represents the effect of exposing similar cells for 30 minutes to anesthetic No. 3. These cells failed to metabolize when the medium was changed; yet the cells looked healthy in various parts of the preparation. Our early estimations of cytotoxicity, therefore, were questionable, and improved methods were sought.

McLimans *et al.*,[7] in work on suspension cultures, indicated that, as a result of permeability changes, degenerating cells stain readily with trypan blue. Hanks and Wallace[8] found that eosin Y helped to differentiate viable from non-viable cells. An attempt was made in this investigation to adapt viability-staining techniques to evalu-

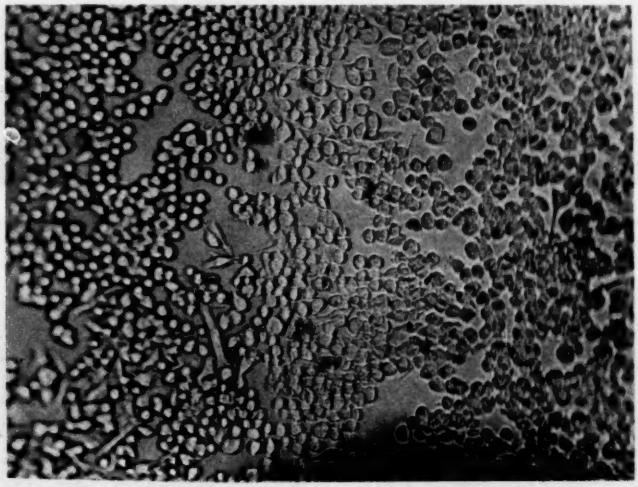

FIG. 3.—Control, HeLa cells exposed for 30 minutes to Hanks' BSS, made up at pH 4.0. Only an occasional stained cell is visible.

ate the comparative cytotoxic effects of the various anesthetics. Both trypan blue and eosin Y were tried. Trypan blue seemed to give a sharper differentiation and was adopted as the stain of choice: non-viable cells appeared blue on microscopic examination, while viable cells appeared colorless. Figures 3–5 illustrate the use of the stain in estimating the comparative quantitative cytotoxicity of the anesthetics.

Our results indicate that the anesthetics studied vary quantitatively in their cytotoxic effects. One of the anesthetics showed considerable cytotoxicity within 30 minutes, while with the others some viable cells remained even after 2 hours. However, it must be emphasized that, while differences were noted in the cytotoxic effects by the various commercial preparations of anesthetics, the results were obtained only in vitro and on two cell preparations. It should be borne in mind that there is as yet no way of translating in vitro results to in vivo application. The observations made in the present investigation are encouraging in demonstrating progress toward a quantitative technique, although much more work remains to be done. Additional methods are being

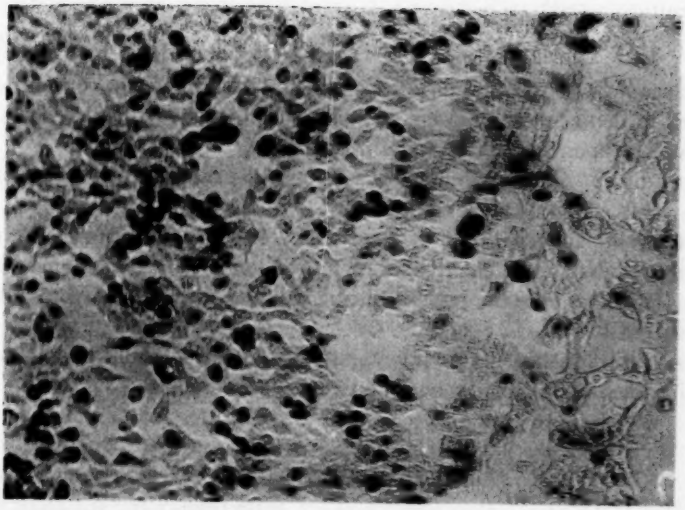

FIG. 4.—HeLa cells exposed to anesthetic No. 6 for 30 minutes. There are many stained cells, indicating appreciable cytotoxicity.

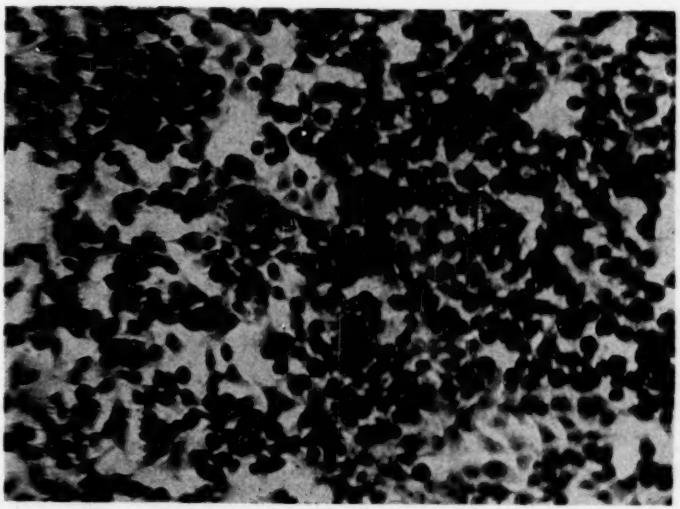

FIG. 5.—HeLa cells exposed to the anesthetic No. 3 combination for 30 minutes. Almost all the cells are stained, indicating marked cytotoxicity of the anesthetic after 30 minutes' exposure of the cells.

explored with a view to improving both qualitative and quantitative determinations in tissue culture.

SUMMARY

1. Nine commercial preparations of local dental anesthetics, in concentrations commonly used in dental practice, were compared as to their cytotoxic effects on monolayer cultures of monkey kidney and HeLa cells.

2. Trypan blue, 0.5 per cent aqueous solution, was employed to differentiate viable from non-viable cells following contact with the anesthetics.

3. The commercial preparation of anesthetic No. 3 was found to be the most cytotoxic; anesthetic No. 6 was slightly less cytotoxic; of the remaining anesthetics, anesthetics Nos. 1 and 2 were the least cytotoxic. All anesthetics studied were completely cytotoxic in 2–3 hours.

4. Tissue-culture techniques as investigative procedures should be explored further to determine their usefulness in quantitative biologic studies.

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Microradiographic and Polarized-Light Studies of Artificially Produced Lesions

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Enamel lesions, produced in vitro under different conditions, have repeatedly been compared with carious lesions by means of microradiographic and polarized-light microscopic procedures. Coolidge, Besic, and Jacobs[1] obtained initial caries-like lesions with an unbroken, glossy surface (subsurface lesions) by exposing intact enamel to buffers in the pH range of 4.0–5.5 containing high concentrations of calcium and phosphate. Buffers containing low concentrations of calcium and phosphate dissolved the enamel surface and produced cavitations. Hals, Morch, and Sand,[2] who employed 0.5 M lactate, obtained both subsurface lesions and cavitations in the pH range 4.1–5.3 and only cavitations when the pH ranged from 3.5 to 4.1 and from 5.3 to 5.7. Upon prolonged exposure to acid, the enamel surface would eventually dissolve and turn subsurface lesions into cavitations. Darling[3] produced subsurface lesions by incubating intact enamel with acidogenic organisms in lactose broth or by exposure to 0.075 per cent lactic acid. Similar lesions were obtained by Forziati, Losee, Pigman, Jennings, and Lawson,[4] who used Pigman's[5] artificial mouth and by Newbrun, Brudevold, and Mermagen,[6] who incubated teeth in saliva-glucose mixtures. The latter investigators also found that 1 M lactate in the pH range 3.7–5.1 produced cavitations simulating an advanced stage of caries.

In the present study an attempt was made to determine conditions which would favor the in vitro development of subsurface lesions and cavitations. Intact enamel was exposed to buffers of different pH and concentrations, and the lesions produced were examined by polarized-light microscopy and microradiography by means of the procedures employed in previous studies of sound and carious enamel.[7, 8]

MATERIALS AND METHODS

Batches of three or more intact teeth, covered with wax except for a portion of the buccal, proximal, or distal enamel surface, were exposed to 100 ml. of acetate buffer until there was visual evidence of etching. The buffers, which were renewed once a week, had a pH of 4.0, 4.5, and 5.0 and molar concentrations of 0.1, 0.01, and 0.001. The change in pH of the solutions did not exceed 0.1 pH unit.

Ground sections of the lesions were subjected to retardation measurements and radiographic assessment according to previously described procedures.[7] Since the results obtained on different lesions were essentially similar, the findings on only one representative lesion are reported in detail.

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RESULTS

The periods of time needed to produce visible lesions, and the type of lesion produced under the different conditions of this experiment are given in Table 1.

At each pH level an increased period of time was needed to produce a lesion when the molarity of the buffer was decreased. Increasing the pH of the buffer also lengthened the time for the lesions to appear. The type of lesions formed likewise depended on the pH and buffer concentration. The lesions were grouped in one of the two following categories: (1) Cavitation, which involved visible loss of part of the surface enamel. Subsurface enamel was invariably more affected than the surface enamel. (2) Subsurface decalcification covered by an apparently intact surface layer of enamel.

It will be noticed from Table 1 that, at pH 4.0, cavitations were generally produced by the 0.1 and 0.01 M buffers, while surface and subsurface lesions were produced in

TABLE 1

TYPE OF LESION PRODUCED BY EXPOSURE OF TEETH TO 0.1, 0.01, AND 0.001 M ACETATE BUFFERS pH 4.0, 4.5, AND 5.0

pH	MOLARITY	TIME	NO. OF TEETH EMPLOYED	TYPE OF LESION	
				Cavitation	Subsurface
4.0	0.1	1–2 days	8	8	.....
4.0	.01	4 days–1 week	7	7	.....
4.0	.001	10–12 weeks	7	4	3
4.5	.1	1–2 weeks	8	1	7
4.5	.01	8 weeks	8	2	6
4.5	.001	12 weeks	8	2	6
5.0	.1	12 weeks	8	2	6
5.0	.01	12 weeks	8	1	7
5.0	0.001	15 weeks	8	.....	8

about equal numbers by the 0.001 M buffer. At higher pH, mainly subsurface lesions were produced, regardless of the concentration of the buffer.

Figure 1 shows a microradiograph of a lesion classified as type 1 and produced by exposing a first molar for 1 week to 0.01 M acetate, pH 4.0. Decalcification is pronounced in both surface and subsurface enamel, but the relative resistance of the surface enamel to dissolution can be clearly seen. At regular intervals there appears to be increased dissolution of the external enamel, suggesting periodic variations in the structure or composition.

Figure 2 shows a type 1 lesion produced by exposure to 0.01 M buffer, pH 4.0, for 5 days. This lesion is similar to that in Figure 1 except that a layer of relatively undecalcified enamel can be seen between the external surface and the internal border of the lesion.

Figure 3 shows a microradiograph of a type 1 lesion produced by exposure to 0.001 M acetate, pH 4.0, for 12 weeks. The lesion is shallower than that shown in Figure 1, and the surface enamel is better preserved; otherwise, the lesion appears radiographically similar.

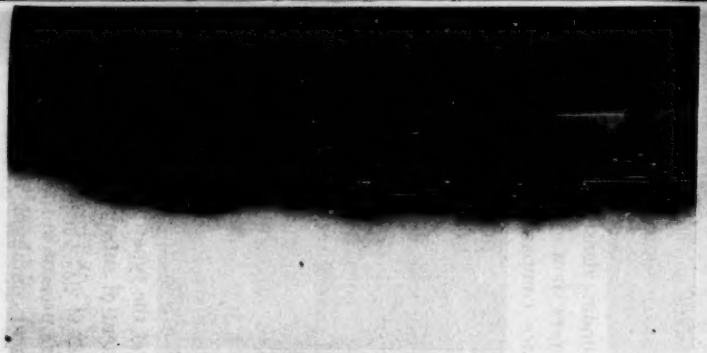

FIG. 1

FIG. 3

FIG. 2

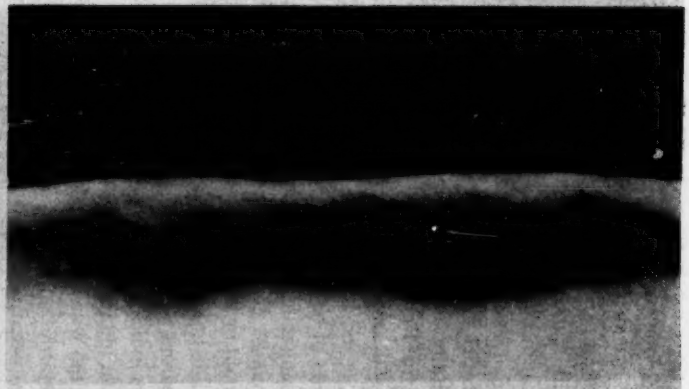

FIG. 4

FIG. 1.—Microradiograph of an enamel lesion produced by etching with 0.01 M acetate buffer, pH 4.0, for 1 week. (Mag. ×100.)
FIG. 2.—Microradiograph of an enamel lesion produced by etching with 0.01 M acetate buffer, pH 4.0, for 5 days. (Mag. ×100.)
FIG. 3.—Microradiograph of an enamel lesion produced by etching with 0.001 M acetate buffer, pH 4.0, for 12 weeks. (Mag. ×100.)
FIG. 4.—Microradiograph of an enamel lesion produced by etching with 0.01 M acetate buffer, pH 5.0, for 15 weeks. (Mag. ×100.)

Figure 4 shows a type 2 lesion obtained by exposure to 0.01 M acetate, pH 5.0, for 15 weeks. The surface enamel appears to be preserved in greater thickness than in the previous lesion, and the decalcification of subsurface enamel is less pronounced. Decalcification is marked along and outward from the striae of Retzius, as is commonly found in carious lesions.

Figure 5, *a*, shows a microradiograph of a type 2 lesion produced by exposure to 0.01 M acetate, pH 4.5, for 8 weeks. Again the surface enamel is relatively well preserved, and dissolution of subsurface enamel is irregular and accentuated along and out from the striae of Retzius. Polarized-light photomicrographs of the specimen imbibed in cinnamon oil and in iodide having the same refractive index (1.620) are shown in Figure 5, *b* and *c*.

The zones of birefringence described in carious lesions[8] can be seen in Figure 5, *b*. In the polarizing microscope zone *1* appeared as a green area at the junction of normal and carious enamel. Zone *2* was reddish, and zone *3* was light green. In zone *4* the striae of Retzius could be seen ranging in color from sky blue to green.

The iodide imbibition changed the colors of birefringence. Figure 5, *c*, shows that the zones apparent with cinnamon oil imbibition have disappeared.

A region indicated by the arrow in Figure 5, *a*, was subjected to retardation measurements when imbibed both in cinnamon oil and in iodide, refractive index 1.620. Areas were selected which were located in the zones seen in Figure 5, *b*. Table 2 shows that positive retardation was obtained in zone 2 with cinnamon oil imbibition, while the other zones gave negative values. Imbibition with iodide produced negative retardations in the entire lesion, and they were consistently greater than those obtained with cinnamon oil. Additional measurements of each area, employing iodide solutions of different refractive indices, showed that the figures given in Table 2 represented intrinsic retardations.

The intrinsic retardations of the lesion and a corresponding densitometric tracing of the microradiograph are plotted in Figure 6. The two curves show excellent correlation, both demonstrating slight decalcification of the surface compared with subsurface enamel and marked decalcification along the striae of Retzius.

DISCUSSION

The lesion obtained from weak buffers after exposure for 8 or more weeks appeared similar to caries with regard to both X-ray absorption and birefringence in different media. Etchings produced by strong buffers at low pH did not generally extend so far into the enamel as did carious lesions, and the affected enamel was more decalcified. Newbrun *et al.*,[6] employing 1 M buffers, observed that the surface enamel was severely affected and that it was completely dissolved in some cases, producing deep cavitations not seen in the present study. Hals *et al.*[2] showed that 0.5 M buffer also occasionally caused complete loss of surface enamel. However, subsurface lesions occurred much more frequently than in the Newbrun study. It is evident from the foregoing that the concentration of the buffer has an important bearing on the type of lesion produced. The weaker the buffer, the more likely is the development of subsurface lesions. This conclusion may seem contrary to the findings of Coolidge *et al.*[1] They obtained cavitations when low concentrations of calcium phosphate were present in the acid solutions, but only "loss of opacity" (subsurface lesions) with acid containing high concentra-

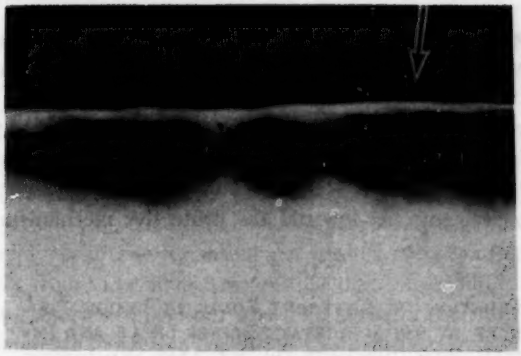

FIG. 5a

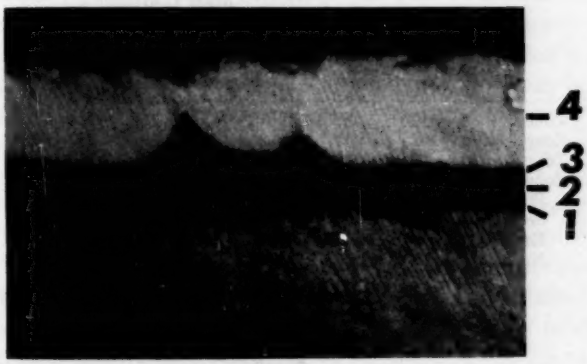

FIG. 5b

FIG. 5c

FIG. 5.—*a*, Microradiograph of an enamel lesion produced by etching with 0.01 M acetate buffer, pH 4.5, for 8 weeks. *b*, Photomicrograph of same specimen as shown in *a*; imbibition with cinnamon oil (R.I. 1.618) (Mag. ×100.) Zones indicated by numbers. *c*, Same specimen as in b; imbibition with iodide solution (R.I. 1.620). (Mag. ×100.)

tions of calcium phosphate. However, since calcium and phosphate ions in the acid would tend to counteract the process of decalcification, the effect of buffer concentration was obscured in this study.

Aside from buffer concentration, the pH materially affected the rate of development of the lesions. The dissolution of surface enamel at high pH was so slight, even in the presence of a marked subsurface lesion, that it could be detected radiographically only by densitometric measurements. At low pH the protective mechanism of the surface

TABLE 2

RETARDATION OF DIFFERENT ZONES IN LESION PRODUCED BY 0.01 M ACETATE BUFFER pH 4.5 AND IMBIBED WITH CINNAMON OIL R.I. 1.618 AND IODIDE SOLUTION R.I. 1.620

AREAS EXAMINED	BEREK COMPENSATOR RETARDATION (MU)	
	Cinnamon Oil	Iodide Solution
Zone 1	−185	−194
Zone 2	+ 41	−153
Zone 3	−193	−159
Zone 4		
First striae of Retzius	−109	−149
Second striae of Retzius	−157	−161
External surface	−151	−210
Normal enamel	−293	−386

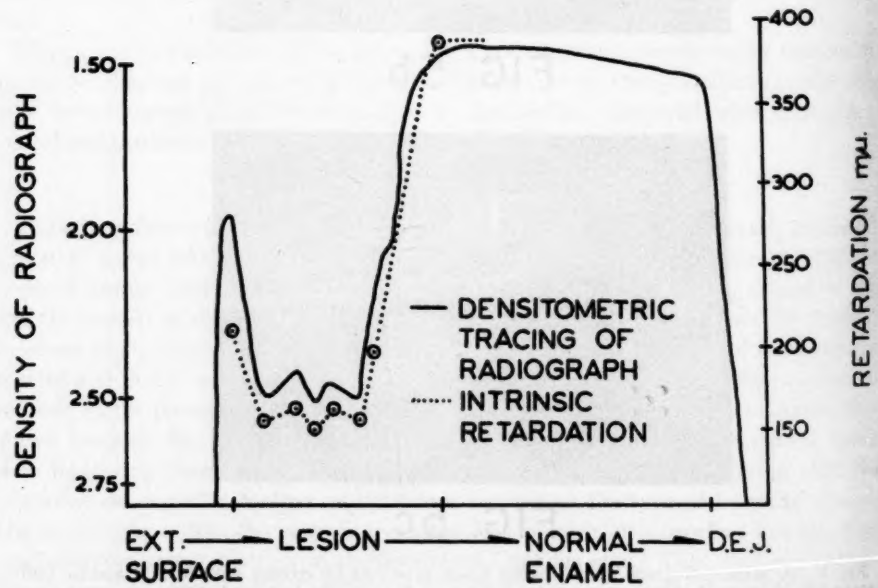

FIG. 6.—Comparison of intrinsic retardation of specimen shown in Fig. 5, *c* (imbibition with iodide solution R.I. 1.620), and densitometric tracing of radiograph.

seemed to break down. Apparently the rate of dissolution of the surface enamel under this condition was greater than the rate of diffusion into underlying enamel, and cavitation resulted.

It seems remarkable that the surface enamel which was in direct contact with the buffers was decalcified at a slower rate than the subsurface enamel. The finding of Isaac that external enamel is more resistant to acids than internal enamel[9] appears to account for this pattern of solubility. It is of considerable interest that the greater resistance to acids afforded by surface than by subsurface enamel persists after all organic material has been removed from the enamel,[9] suggesting that the solubility-reducing mechanism is tied in with the inorganic portion. The high concentration of fluoride and low concentration of carbonate in surface compared to subsurface enamel are known contributory factors,[10, 11] and other trace elements may be involved.

It is likely that the organic matrix in the enamel may facilitate, rather than resist, decalcification, since both etched and carious lesions tend to advance along structures in the enamel known to be high in organic material—the interprismatic substance, the striae of Retzius, and the Hunter-Schreger lines.[12] The finding that a portion of the organic material of enamel is soluble in acid[13] suggests that voids may be created which will permit diffusion of the decalcifying agent.

Several workers have suggested that a combined process of decalcification and recalcification occurs in the carious lesions. The conditions of the present study did not permit recalcification, and yet carious-like lesions were produced. The lesion reproduced in Figure 2 is particularly interesting because of the presence of a narrow area of radiopaque material separated from the surface enamel and the internal enamel by decalcified zones. Such areas are often seen in carious enamel lesions and have been attributed to recalcification. It is more likely that they repersent residues of relatively insoluble enamel and that their occurrence is related to the tendency of fluoride to be retained by enamel undergoing decalcification[9] and the opposite tendency of carbonate to be preferentially dissolved.[11] These reactions, which are known to take place in carious enamel,[14, 15] may render zones of enamel insoluble to the extent that they may be bypassed by the decalcifying agent.

In conclusion, it may be stated that the present findings strongly point to acid as being the causative factor in dental caries. It is unlikely that highly concentrated acids are involved. It is rather suggested that lesions are produced by low concentrations of acid, perhaps in the range of 0.01–0.001 M. Proteolysis may, of course, also take place in the initial lesion, but there is no evidence from the present study that it is a significant or necessary etiological factor. The good agreement between the retardation measurements and X-ray densities suggests that the polarizing microscope is well suited for studying experimental enamel caries.

SUMMARY

1. Acid-etched lesions were produced by exposing enamel to acetate buffers at a pH of 4.0, 4.5, and 5.0 and a concentration of 0.1, 0.01, and 0.001 M.

2. Plane-parallel ground sections of the etched enamel, investigated by polarizing-light microscopy and microradiography, revealed that different types of lesions were produced, depending on the pH, the strength of the buffer, and the time of acid exposure.

3. Subsurface lesions similar to incipient carious lesions were produced by the weaker buffers at all pH levels.

4. Surface lesions simulating an advanced stage of carious lesions were produced by stronger buffers and at low pH's.

5. Polarized and microradiographic examination of the subsurface lesions revealed (*a*) partial decalcification of the external surface of the enamel; (*b*) variable decalcification in different areas of the same sections; (*c*) no indication of hypercalcification in different types of acid-etched lesions; and (*d*) progress of the lesion along the interprismatic substance and the striae of Retzius.

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Studies of Corynebacterium *Isolated from Non-exposed Human Pulp Canals*

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I. Morphological and Physiological Characteristics

As early as 1894, W. D. Miller[1] reported isolating club-shaped, barred, and beaded forms of bacteria from pulp canals. In 1919 Henrici and Hartzell[2] listed diphtheroid bacilli among the bacteria isolated from pulp tissue. Recently, Brown and Rudolph[3] found that members of the genera *Streptococcus* and *Corynebacterium* were the most frequently occurring bacteria in unexposed root canals of teeth with pathological pulps. Smith, Thomassen, and Sweet[4] cultured 41 strains of *Corynebacterium* from root canals in a study of 90 such teeth. The selection of teeth and the techniques used precluded contamination of the specimens by normal oral flora. Because of the high incidence of strains of *Corynebacterium*, it was felt that further study of these organisms would be of value.

The isolation of strains of *Corynebacterium* from both human and animal sources has been reported.[5] However, descriptive studies are too scant to allow comparison of the organisms isolated from different sources. Murray and Morse[6] used habitat relationships to construct a key for species identification in Bergey's *Manual of Determinative Bacteriology*. This paper describes the morphological and physiological characteristics of 14 strains of *Corynebacterium* isolated by Smith *et al.*[4]

MATERIALS AND METHODS

The aseptic methods used to obtain pulp contents from which strains of *Corynebacterium* were cultured have been described by Smith *et al.*[4] Pure cultures of the bacteria were obtained by transferring isolated colonies from blood agar plates (blood agar base* plus 5 per cent defibrinated sheep blood) to semisolid medium,[4] incubating at 37° C., and replating them. This procedure was repeated three times. The blood agar plates were observed both by the unaided eye and under the low power of the microscope to determine the type of hemolysis produced. The bacteria, which on primary isolation grew deep in brain-heart infusion plus 10 per cent ascitic fluid medium containing 0.1 per cent agar and only under the surface in poured blood agar plates, grew on the surface of solid media and in broth not containing 0.1 per cent agar after repeated serial transfer with heavy inocula. The bacteria were streaked on blood

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* Tryptose Blood Agar Base, Difco Laboratories, Inc.

tellurite (5 ml. bacto-tellurite blood solution added to 100 ml. bacto-dextrose proteose No. 3 agar) plates for observation of growth on this medium.

Measurements of organisms from 48- to 72-hour-old cultures of brain-heart infusion plus 1 per cent yeast extract broth were made by means of a calibrated ocular micrometer; smears of the bacteria at the time of primary isolation and after being trained to grow aerobically were stained by Gram's method; the presence of metachromatic granules was determined by observing smears stained with Pugh's acid toluidine-blue; smears of 1-, 2-, 3-, and 4-week-old cultures were stained with malachite green and counterstained with safranine, then examined for the presence of spores; cultures were stained by the Ziehl-Neelsen method and observed for acid-fastness; throughout these studies the cultures were constantly observed for the appearance of pigment; 24-, 48-, and 72-hour-old cultures were examined for motility by the hanging-drop method. The presence of catalase was determined by observing the evolution of gas bubbles after adding a drop of 3 per cent hydrogen peroxide to a bacterial colony which had been transferred to a glass slide. Smears of organisms grown in media containing 10 per cent ascitic fluid were stained by Hiss capsule stain and observed for the presence of capsules.

In preparation for metabolic tests, serial transfers with heavy inocula of each strain were made into carbohydrate-free medium* until eugonic growth occurred. Fermentation tubes containing 10 ml. of the medium were sterilized by autoclaving 15 pounds for 20 minutes.

To this basal medium, aqueous solutions of the various carbohydrates were added aseptically to make a final concentration of 1 per cent, and two tubes of each were inoculated with each of the strains of *Corynebacterium*. The pH was determined by the glass-electrode pH meter† after 48 and 96 hours' incubation at 37° C. Controls of uninoculated carbohydrate media and of inoculated basal medium were tested simultaneously. The carbohydrate media controls retained a pH of 7.2, and none of the inoculated non-carbohydrate cultures developed a pH below 6.9. Therefore, cultures grown in carbohydrate media which developed a pH below 6.9 were considered to form acid. The methods used for the methyl-red test, Voges-Proskauer test, reduction of nitrates, hydrogen sulfide production, liquefaction of gelatin, indol production, and reaction on litmus milk are described by Wilson and Miles.[5]

For heat-inactivation tests, Wassermann tubes containing 3 ml. of brain-heart infusion plus 1 per cent yeast extract and 0.1 per cent agar were heated at various temperatures in a thermostatically controlled water bath. One loopful of a 72-hour culture of each of the 14 strains was inoculated into a tube of the pre-heated medium, which was immediately returned to the water bath for 10 minutes, after which it was incubated at 37° C. for 5 days. At this time the cultures were observed for visible growth. A series of unheated controls was incubated simultaneously; growth occurred in all of these.

RESULTS

Surface colonies on blood agar plates after 48 or 72 hours' incubation at 37° C. were glistening, round, smooth, low-convex, and ½–1 mm. in diameter. Black, low,

* Tryptose, 17.0 gm.; Phytone (Baltimore Bio. Lab., Inc.), 3.0 gm.; sodium chloride, 5.0 gm.; dipotassium phosphate, 2.5 gm.; H_2O q.s., 1.0 liter. Final pH, 7.2.

† Model G, Beckman Instruments, Inc.

convex colonies, 2–4 mm. in diameter, developed on blood tellurite agar plates. A characteristic garlic odor was observed as growth of the organisms developed.

Organisms of all strains measured 0.5 μ wide. The length of some strains varied between 2 and 3 μ and others between 1.5 and 4 μ. All the organisms were Gram-positive and, on primary isolation, appeared as beaded rods which resembled short-chain streptococci as described by Wilson and Miles.[5] After several transfers on laboratory media the organisms became pleomorphic and were often club-shaped, bipolar, or barred, as well as beaded. The arrangements of postfission grouping were pallisade and "Chinese character writing." All strains had metachromatic granules, none formed spores, and none were acid-fast. Strains 5 and 10 developed yellow pigment. Strains 2, 5, and 10 showed an indefinite halo, but no strain developed a definite capsule.

In accordance with *Bergey's Manual of Determinative Bacteriology*,[6] the bacteria in this study were placed in the genus *Corynebacterium* because, in addition to the typical morphological characteristics and postfission groupings, all the strains were non-motile and produced catalase.

Each of the 14 strains showed a different pattern of biochemical reactions. No visible gas was produced by any of the strains when cultured in various carbohydrate media. Strain 2 was the most active biochemically; strains 7, 10, and 13 were the least active. Levulose, the most frequently utilized carbohydrate, was metabolized by 12 of the 14 strains (Table 1). These characteristics, which are often used for species classification, showed the bacteria to be a heterogeneous group.

The bacteria of this group are more heat-resistant than are the type species of the genus, *Corynebacterium diphtheriae*, which is killed by heating at 58° C. for 10 minutes.[5] The strains most sensitive to heat inactivation, Nos. 1 and 2, resisted heating at 60° C. for 10 minutes, but not at 70° C. The most resistant strains, Nos. 3, 5, 11, and 13, resisted heating at 80° C. but not at 90° C. for 10 minutes (Table 2).

SUMMARY

The morphological characteristics, staining reactions, typical colony formation on blood agar and tellurite blood agar, and biochemical reactions of 14 strains of bacteria isolated from non-exposed human pulp canals were studied.

Each of the strains was classified as a member of the genus *Corynebacterium*. The biochemical reactions of each strain differed, showing this to be a heterogeneous group of bacteria.

Death of the strains of bacteria by heating occurred at temperatures of 70°–90° C. for 10-minute periods—a relatively greater resistance than that reported for the type species, *C. diphtheriae*.

II. Pathogenicity*

Attention has long been focused on the pathological condition caused by toxin-producing strains of *C. diphtheriae*. More recently, Pappenheimer[7] reported that completely non-toxigenic strains were not entirely lacking in pathogenicity. The avirulent C_4 and C_7 strains produced purulent local lesions resembling those caused by staphylococci when injected into the skin of laboratory animals. *Corynebacterium ovis* produces a filterable toxin and is also a pyogenic organism. Other members of the genus

*Some of the material included in this study was presented at the thirty-seventh meeting of the International Association for Dental Research, March 19–22, 1959, at San Francisco, California.

TABLE 1*

BIOCHEMICAL REACTIONS OF 14 STRAINS OF *Corynebacterium* ISOLATED FROM UNEXPOSED HUMAN ROOT CANALS

	Strain													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Dextrose	–	4.1†	–	–	5.2	6.2	–	5.2	–	–	–	5.0	–	5.3
Lactose	–	4.3	–	–	5.2	–	–	6.2	–	–	–	–	–	–
Maltose	6.1	4.4	6.3	–	5.3	6.3	–	5.0	6.5	–	6.5	5.3	–	–
Xylose	6.8	6.6	–	6.7	–	–	–	–	–	–	–	–	6.8	–
Arabinose	6.7	5.0	–	6.5	–	–	–	–	–	–	–	–	–	–
Raffinose	–	4.4	–	–	–	–	–	–	–	–	–	–	–	–
Levulose	4.9	3.4	5.5	4.9	4.9	5.5	6.4	5.1	6.5	–	6.3	5.0	–	4.9
Galactose	–	6.4	–	–	5.3	–	–	6.8	–	–	6.7	–	–	–
Saccharose	5.3	4.1	–	4.0	6.4	4.1	–	5.2	–	–	–	–	–	5.8
Mannitol	–	4.4	–	6.8	–	–	–	–	–	–	–	–	–	–
Glycerol	–	–	–	–	–	6.5	–	5.5	–	–	–	–	–	–
Sucrose	5.5	4.1	–	5.3	5.1	–	–	5.3	–	–	–	–	–	5.6
Dextrin	–	6.7	–	–	6.4	–	–	–	–	–	–	–	–	–
Saccharin	6.7	–	–	–	–	–	–	–	–	–	–	–	–	6.7
Dulcitol	6.8	5.1	–	–	–	–	–	–	–	–	–	–	–	6.8
Salicin	–	4.6	–	6.8	–	–	–	–	–	6.8	–	–	–	–
Inulin	6.7	4.1	–	6.8	–	–	–	–	–	–	–	–	–	6.7
Methyl red	–	+	–	–	+	–	–	+	–	–	–	–	–	–
Voges-Proskauer	–	–	–	–	+	–	–	+	+	–	–	–	–	–
Nitrates reduced to nitrites	–	–	–	–	–	–	–	+	–	–	–	+	–	–
H_2S	–	+	–	+	–	–	–	–	–	–	–	–	–	–
Liquefaction of gelatin	+	–	–	+	+	–	–	–	–	+	–	–	–	+
Indol	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Litmus milk	P	AC	–	P	P	–	–	A	–	–	–	P	–	P
Type of hemolysis	b	b	b	a	a	b	b	b	b	nh	b	b	b	b

* + = positive; – = negative; a = alpha; b = beta; nh = non-hemolytic; P = peptonization; A = acid; C = curd.
† pH developed by cultures grown in the carbohydrate media.

TABLE 2*

RESISTANCE OF *Corynebacterium* ISOLATED FROM UNEXPOSED HUMAN ROOT CANALS TO VARIOUS TEMPERATURES FOR PERIOD OF 10 MINUTES

Strain	Temperature (° C.)				
	50	60	70	80	90
1	+	+	–	–	–
2	+	+	–	–	–
3	+	+	+	+	–
4	+	+	+	–	–
5	+	+	+	+	–
6	+	+	+	–	–
7	+	+	+	–	–
8	+	+	+	–	–
9	+	+	+	–	–
10	+	+	+	–	–
11	+	+	+	+	–
12	+	+	+	–	–
13	+	+	+	+	–
14	+	+	+	–	–
c	+	+	+	+	+

* + = growth of organisms; – = no growth; c = control.

Corynebacterium which produce localized lesions are *C. pyogenes, C. equi, C. renale, and C. murium*.[8]

The purpose of this study was to determine the pathogenicity of strains of *Corynebacterium* inoculated intraperitoneally into mice and intracutaneously into normal and X-ray-irradiated rabbit abdomens.

MATERIALS AND METHODS

Preparation of inocula.—The cultures used throughout this study consisted of 14 strains of *Corynebacterium* isolated from nonexposed human pulp canals by Smith *et al*.[4] Inocula for animal experiments were prepared as follows, and in each case 0.1 ml. quantities were used.

1. Viable Bacteria.—Seventy-two-hour-old cultures incubated at 37° C. in brain-heart-infusion-yeast extract (BHI-YE) broth were used as inocula.

2. Heat-killed Cells.—Seventy-two-hour-old cultures incubated at 37° C. in BHI-YE broth were heated at 90° C. for 30 minutes, then tested for sterility.

3. Acetone-extracted Cells.—These were prepared according to the method described by Baker, Sommer, Foster, Meyer, and Meyer.[9]

4. Culture Filtrates.—Filtrates were obtained from 1-, 2-, and 4-week-old cultures incubated at 37° C. in BHI-YE, using the Swinny filter adapters for Seitz filter disks.* The filtrates were tested for sterility.

Inoculation of mice.—A series of 10 NAMRU[10] mice were inoculated intraperitoneally with 0.1 ml. of each of the 14 strains of living organisms. Five mice were inoculated with 0.1-ml. quantities of sterile incubated medium as controls. Five mice were inoculated with heat-killed organisms of strain 14. The mice were observed daily over a period of 2 weeks for signs of toxic reactions. After this interval, all survivors were sacrificed by injecting 0.1 ml. aqueous solution containing 5 mg. of sodium pentothal.† The mice were moribund 2 minutes after the injection. The animals were opened by midline incision extending the length of the abdomen and thorax. Oblique incisions were made from the midline incision to each of the four legs. The peritoneum and internal organs were inspected for gross pathological characteristics.

Hematoxylin-eosin–stained sections, 8μ thick, of selected livers and spleens, showing typical gross pathological characteristics and pustules excised from the peritoneum, were examined for histopathologic changes.

Experiments with rabbits.—New Zealand white male rabbits 10–11 weeks old were used for intracutaneous inoculation. The abdomens were prepared by shaving and treating with lanolin for 4 days to allow healing of any irritation resulting from the shaving. Each abdomen was marked off into 15 areas, one for each of the 14 strains of *Corynebacterium* and one for injection of 0.1 ml. of sterile medium as a control.

Eight rabbits were inoculated intracutaneously with viable organisms. The diameters of the papules and macules which developed were measured daily. The time of resolution, defined as the time at which the lesion became so diffuse that no definite outline could be determined, was observed. One animal from this group was sacrificed 72 hours after injection. Hematoxylin-eosin–stained sections taken from each lesion, 8 μ thick, were examined for histopathology.

* Republic Seitz Filter Corporation.

† Abbott Laboratories.

Six rabbits were anesthetized by intravenous injections of 45 mg. sodium pentobarbital.* Each rabbit was held immobile on an animal board, and the abdomen was divided into 16 equal sections comparable to the areas exposed in a 16-film full-mouth X-ray. A condenser r meter† with a 25-r chamber measured the X-ray dosage at target point to be 2.0 r per second. Each area was exposed for 5 seconds, at a target distance of 8 inches, to 10 r of X-ray, or a total of 160 r over the abdomen. The X-ray unit‡ was used at 60 KVP, 10 milliamperes, with only the inherent filtration of the machine, allowing both hard and soft rays to reach the animals. Forty-eight hours after irradiation viable organisms were injected as described previously.

The diameters of the lesions were measured daily, and the time of resolution was observed. One animal from this group was sacrificed 72 hours after injection. Hematoxylin-eosin–stained sections taken from each lesion were examined for histopathologic changes.

Six rabbits were inoculated intracutaneously with heat-killed bacteria. The diameters of the lesions which developed were measured daily. The time of resolution was observed. Seventy-two hours after inoculation, one animal from this group was sacrificed. Hematoxylin-eosin–stained sections, 8 μ thick, of each lesion were examined for histopathologic changes.

Three rabbits were inoculated intracutaneously with acetone-extracted bacteria. The animals were observed daily for the development of lesions at the sites of inoculation.

Two rabbits were inoculated with filtrates from 1-week-old, 2 from 2-week-old, and 2 from 4-week-old cultures. The animals were observed daily for the development of lesions at the sites of inoculation.

RESULTS

During the 2-week observation period no signs of toxicity were observed in mice which had been injected with the viable strains of *Corynebacterium*. One mouse injected with strain 6 and one with strain 11 died from non-specific causes less than 24 hours after injection. One mouse from the group which had been injected with strain 12 and was sacrificed 24 hours after injection showed no pathological characteristics. At autopsy, pustules were observed in 32 of the 137 mice (23.3 per cent) at the sites of inoculation in the peritoneal wall (Fig. 1). Histopathologic examination showed that these were collections of fibrino-purulent exudate surrounded by a wall of young fibrous connective tissue containing numerous vessels and many polymorphonuclear neutrophils. The peritoneal fat surrounding the encapsulation contained numerous macrophages.

In 69 of the 137 mice (50.4 per cent) a sandy granular deposit was observed on the surface of the spleen and liver, often occurring on the lower surfaces of the various lobes (Fig. 2). No histopathologic characteristics were observed in the spleen. The liver showed foci of mobilization of Von Kupfer cells, lymphocytes, and occasional polymorphonuclear neutrophils. These lesions were discrete and sharply circumscribed (Fig. 3, *A* and *B*). Sections of livers injected with medium alone and with heat-killed

* Nembutal, Abbott Laboratories.

† Victoreen Instrument Company.

‡ Model D, Ritter Company, Inc.

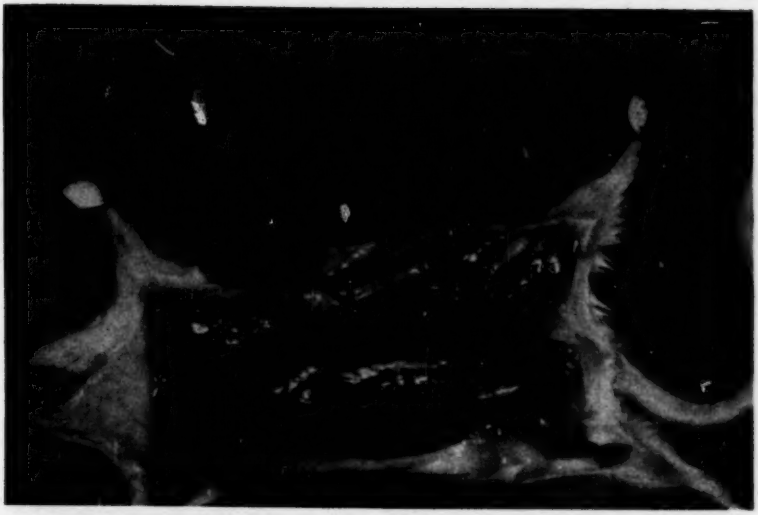


FIG. 1.—Arrow points to pustule which formed in the peritoneal wall of a mouse after injection of strain No. 14 of *Corynebacterium*.

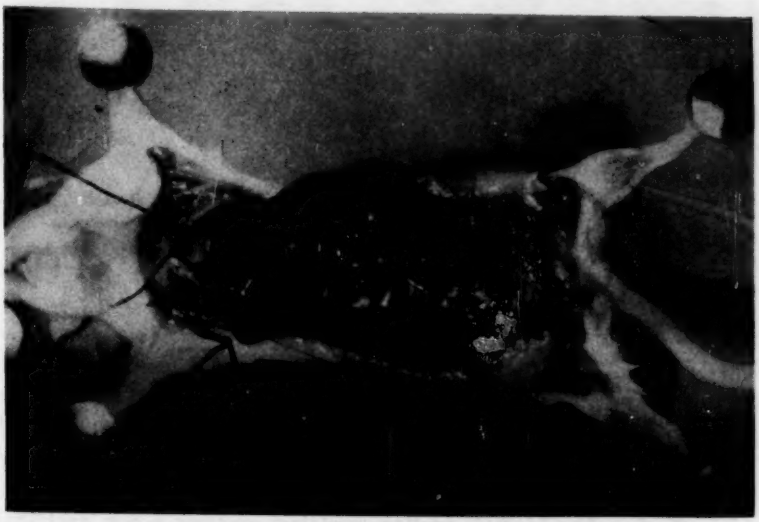
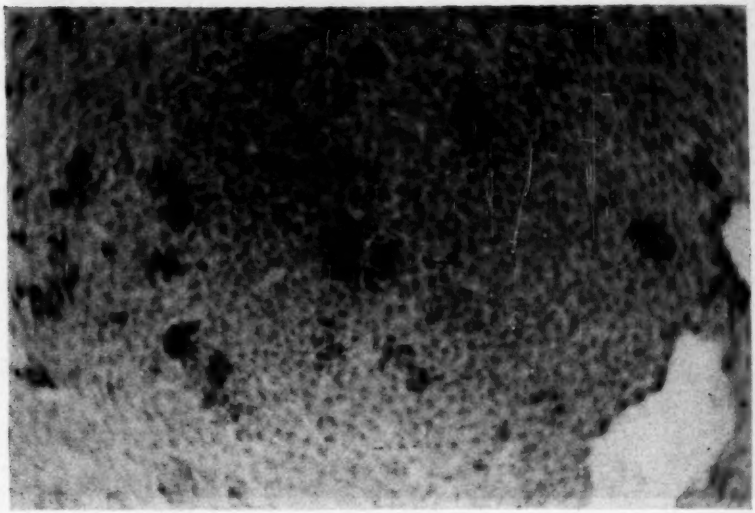
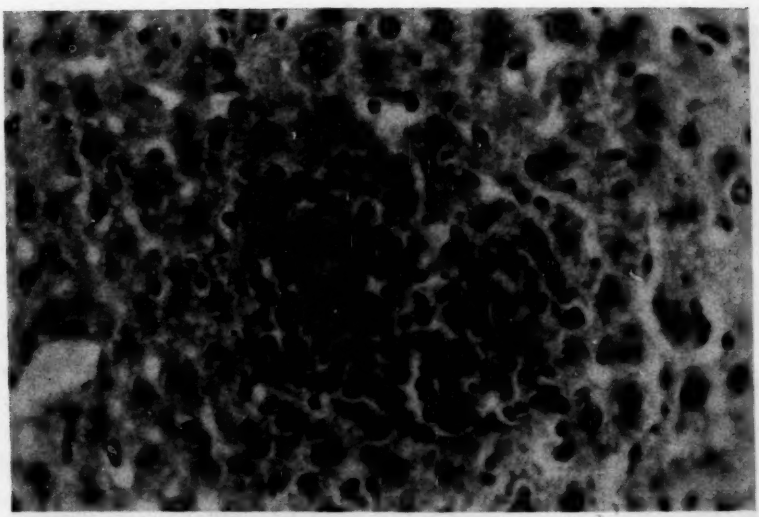


FIG. 2.—Arrow points to a typical granulation observed on the visceral surface of the right lobe of the liver in a mouse 2 weeks after injection of strain No. 6 of *Corynebacterium*.



A



B

FIG. 3.—Foci of Von Kupfer cells, lymphocytes, and occasional polymorphonuclear neutrophils observed in mouse livers 2 weeks after injections of strains of *Corynebacterium*. *A*, a group of lesions (orig. mag. ×100); *B*, a single lesion (orig. mag. ×400).

organisms did not show such lesions. No pustules or deposits were present in any of the control mice (Table 3).

Twenty-four hours after inoculation of strains of *Corynebacterium* intracutaneously into rabbit abdomens, macules from 5 to 12 mm. in diameter developed at the sites of injection. After 48–72 hours, a papule formed in the center of each macule, and the lesions reached the maximum diameters. The macules ranged from 8 to 13 mm. and the papules from 5 to 8 mm. in diameter. These lesions resolved without draining. The time of resolution varied from 4 to 19 days. Hematoxylin-eosin–stained sections of the 72-hour lesions produced by the 14 strains of *Corynebacterium* exhibited different stages of resolution, but all showed a similar histologic pattern. Each lesion consisted of a collection of necrotic debris and disintegrating polymorphs, sharply cir-

TABLE 3

AUTOPSY FINDINGS OF MICE SACRIFICED 2 WEEKS AFTER INTRAPERITONEAL INJECTIONS OF VIABLE STRAINS OF *Corynebacterium*

	STRAIN														MEDIUM CONTROL	HEAT-KILLED STRAIN MIXTURE
	1	2	3	4	5	6	7	8	9	10	11	12	13	14		
Peritoneal pustule only	3/10*	0/10	0/10	2/10	0/10	0/9	1/10	4/10	0/10	0/10	0/9	0/9	0/10	7/10	0/5	0/5
Liver or spleen lesion only	1/10	8/10	4/10	0/10	0/10	3/9	3/10	0/10	8/10	10/10	6/9	5/9	2/10	4/10	0/5	0/5
Peritoneal pustule and liver or spleen lesion	1/10	0/10	2/10	2/10	2/10	1/9	1/10	3/10	0/10	0/10	0/9	1/9	1/10	1/10	0/5	0/5

* Number of mice showing lesions over number of mice observed.

cumscribed by a wall of young fibrous connective tissue containing numerous vessels and many polymorphs. Beyond this encapsulation was the normal collagenous tissue and voluntary muscle of the abdominal wall (Fig. 4).

When the viable strains of *Corynebacterium* were injected intracutaneously into the abdominal skin of X-ray-irradiated rabbits, fiery-red inflamed papules with sharply raised edges developed at the sites of injection. All the lesions had reached a maximum diameter (6–12 mm.) by 72 hours. The time of resolution ranged from 20 to 40 days, a much longer period than was observed with the non-irradiated animals. These lesions drained spontaneously before healing (Table 4).

Seventy-two hours after inoculation these lesions were in different stages of resolution, but there was a common histologic pattern. Diffuse and multiple collections of necrotic debris and polymorphs showing marked karyorrhexis were observed. Some spotty fibrous tissue proliferation was seen, but there was no tendency toward the formation of walls about the collections of pus. The mature connective tissue adjacent to the widespread collections of exudate showed a marked vasodilatation of large vessels, which accounted for the fiery-red appearance of the lesions.

The lesions in the skin of the irradiated animals were not discrete abscesses such as

were observed in the non-irradiated animals. In contrast, they were poorly circumscribed, multiple, and extended over a much wider area (Fig. 5).

Lesions which developed in response to intracutaneous inoculations of the heat-killed strains of *Corynebacterium* reached their maximum diameter (2–7 mm.) in 24–48 hours. These lesions resembled the papules formed in response to injections of viable organisms on normal rabbits. The time of resolution ranged from 3 to 8 days.

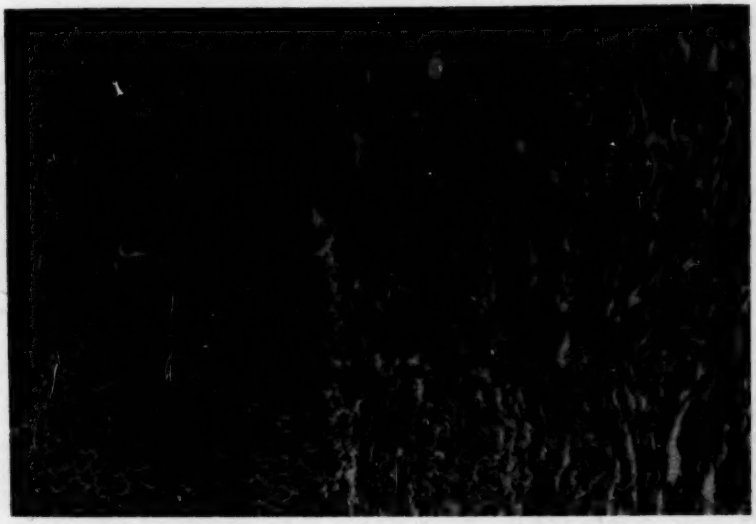


FIG. 4.—Lesion resulting from intracutaneous injections of viable strains of *Corynebacterium* in normal rabbit abdomen. ***From left to right:*** collection of necrotic debris, wall of young fibrous connective tissue, normal tissue.

TABLE 4

DEVELOPMENT OF LESIONS IN RESPONSE TO INTRACUTANEOUS INJECTIONS OF STRAINS OF *Corynebacterium* ON RABBIT ABDOMENS

	Strains													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Viable cells, normal rabbits:*														
Maximum development of lesions in hours	48	48	72	48	72	72	48	48	72	48	48	72	48	48
Maximum diameters of macules in mm.	9	13	9	10	12	8	10	11	13	11	8	8	16	8
Maximum diameters of papules in mm.	7	5	8	7	7	7	8	6	7	7	7	5	8	6
Resolution time in days	18	11	13	15	19	11	9	13	11	10	5	5	4	6
***Viable cells, X-irradiated rabbits:**†*														
Maximum development of lesions in hours	24	48	72	24	24	48	48	48	48	24	48	24	48	48
Maximum diameters of lesions in mm.	11	8	8	8	6	9	8	9	9	7	9	9	9	11
Resolution time in days	40	34	34	40	26	37	40	32	37	20	39	34	39	37
***Heat-killed cells, normal rabbits:**‡*														
Maximum development of lesions in hours	24	24	24	48	24	24	24	24	48	48	48	48	24	24
Maximum diameters in mm.	5	5	5	2	3	5	5	5	6	6	7	6	5	5
Resolution time in days	8	5	4	7	4	4	4	6	6	6	4	4	3	4

* Average figures for 7 rabbits. † Average figures for 5 rabbits. ‡ Average figures for 5 rabbits.

Seventy-two hours after inoculation these lesions were in different stages of resolution but showed a similar histologic pattern. Small diffuse and multiple collections of polymorphs were observed. No fibrous tissue reaction toward a walling-off of the collection of leukocytes was observed. No marked vasodilatation was seen in the connective tissue adjacent to the foci of polymorphs.

Reddened areas of indefinite outline, all of which had disappeared by 72 hours after inoculation, developed in response to some of the filtrates. However, the results were scattered, and positive reactions were not constant with any given strain of organism or with any one incubation period.

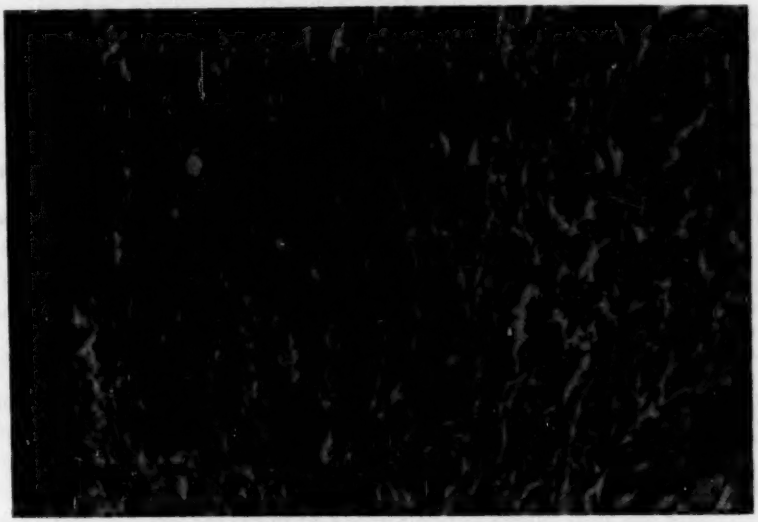


FIG. 5.—Lesion in response to intracutaneous injection of viable strains of *Corynebacterium* in X-ray-irradiated rabbit abdomen. ***From left to right:*** collection of necrotic debris, spotty fibrous tissue with no tendency to wall off the collection of pus.

Response to saline suspensions of acetone-extracted cells was slight. Strains 1, 4, 6, 10, 11, 12, and 13 caused slightly raised, but not discolored, papules without definite outlines, all of which disappeared in 7 days. There was no response to injections of extracts from the other seven strains.

SUMMARY

The pathogenicity of 14 strains of *Corynebacterium* isolated from non-exposed pulp canals was studied.

Of the mice inoculated intraperitoneally with the organisms, 23.3 per cent developed local lesions of the peritoneum at the site of injection, and 50.4 per cent showed gross and histopathologic changes in the liver.

Rabbit abdomens inoculated intracutaneously with the organisms developed localized lesions which healed spontaneously without draining.

Rabbit abdomens exposed to hard and soft roentgen rays before inoculation of the organisms developed fiery-red and sharply raised lesions. These lesions opened and

drained spontaneously before healing. Histopathologically, they were shown to be poorly circumscribed and multiple.

Inoculation of heat-killed cells produced small lesions at the sites of inoculation.

Intracutaneous inoculations of saline suspensions of acetone-extracted cells and of filtrates from 1-, 2-, and 4-week cultures elicited slight reactions. These responses did not occur with any regularity.

The authors wish to acknowledge the technical assistance of dental students Mr. Roland G. Holt and Mr. Guy T. Baker.

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Influence of Artificial Tooth Forms on Bone Deformation beneath Complete Dentures

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Although the cause of alveolar resorption of the edentulous ridge is not known, many people believe that "lateral" forces exerted against these tissues by artificial dentures contribute to bone resorption. A large proportion of these forces is exerted through the occlusal form of the tooth.

The term "lateral" forces is misleading. Forces are either tensile, compressive, or shearing. The first tends to lengthen an object, the second to shorten it, and the third to move one section of an object across another section. The forces of occlusion are complicated and cannot be readily separated. There is definitely a compressive force (which causes a resulting tensile force) and, in lateral excursion, a shearing force. Very often forces which are compressive on the lateral surface of a bone, such as the ascending ramus, may be tensile on the medial surface or vice versa. The shearing force is the most difficult to evaluate on a quantitative basis. Whether this force (which most closely approximates the description of lateral forces) is actually more destructive to the alveolar ridge is at present unknown.

Dempster and Liddicoat[1] showed that compact bone has more compressive strength than tensile strength, but no mention is made of alveolar (non-compact) bone. Evans and Lissner[2] also found the compressive strength of compact bone to be greater than its tensile strength. They did report on the strength of spongy bone but with insufficient data to make valid conclusions, and they made no reference to alveolar bone as such.

In this study the authors wished to ascertain qualitatively whether the so-called non-anatomic or anatomic form of artificial teeth transmitted more energy to the underlying bone. There has been a long-standing philosophical discussion concerning the merits of these tooth forms. The proponents of non-anatomic teeth claim that they are gentler to underlying structures. These non-anatomic teeth do not have interdigitating cusps and do not interfere with one another's free movements in lateral excursions of the mandible. The anatomic tooth has cusps which fit coresponding fossae on the opposing tooth and do interfere to some degree with free lateral movement of the jaw.

Several methods of investigation were considered. All were concerned with the degree of deformation of bone beneath artificial dentures. The first was the use of electric resistance and induction gauges to measure the amount of deformation. This would involve a great deal of preliminary work to locate these gauges. The second was the photoelastic approach, wherein transparent plastic models of phenylformaldehyde resin would be made. These become birefringent in polarized light and demonstrate areas of

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strain.[3] This method was discarded because there is a great deal of doubt as to the applicability of results in a solid model to a complex structure such as bone.

Evans and Goff[4] state that the so-called split-line technique "is of little aid in analysing the mechanical significance of bone architecture."

MATERIALS AND METHODS

The method finally chosen was the brittle-coating technique which was developed by DeForest and Ellis[5] in 1940. This technique* reveals a qualitative and a roughly quantitative analysis of the strain patterns in a body previously deformed. The qualitative analysis can be obtained by spraying one of several brittle lacquers (a particular one chosen to suit the prevailing temperature and humidity conditions) onto the surface of the body to be tested. This is allowed to dry, and the body is loaded in the desired way. Cracks in the lacquer will appear in the area of deformation. These areas can be photographed and compared for an estimate of the qualitative strain produced by the load.

An accurate quantitative analysis can be gained by placing electric strain gauges or mechanical extensometers in the areas of most strain and actually measuring the amount of deformation. The authors became interested in the qualitative analysis at this time but hoped also to gain some insight into quantitative values. The technique* will afford these on a very gross basis.

A series of eighteen human edentulous skulls were obtained from a biological supply house. No data were obtained as to age, sex, or race. Two sets of artificial dentures were constructed for each skull. These were similar in all respects, except that the posterior teeth of one set had anatomical teeth and the other non-anatomic teeth.

The skulls were all coated with a black undercoat supplied by the manufacturer.* This was allowed to dry for 1 hour. The temperature and humidity were recorded, and the proper lacquer to suit these conditions was chosen. This was sprayed onto the skulls and allowed to dry 24 hours. (It was soon noticed that the maintenance of a constant temperature and humidity was essential to the production of readable patterns and enabled the investigator to use the same number lacquer for all tests.)

The dry skull was examined for "craze," which sometimes occurs with a drop in temperature during the 24-hour drying period (Fig. 1), and for drying cracks, which are large and easily identified. If these were apparent, the skulls were cleaned and recoated. If none occurred, the skull was mounted on a specially designed test stand, and the anatomic teeth were set in a centric relationship (Fig. 2).

Centric occlusion was established arbitrarily when the dentures were constructed. That position was chosen where the condyle was in a "normal" relationship to the fossa at the proper vertical dimension. It was maintained for both dentures by a plaster mold. It was then re-established during each testing session by interdigitating the anatomical tooth forms and by matching the interproximal spaces of upper and lower on the non-anatomical forms. The simulated temporomandibular joint limited both the type and the range of movement of the jaws.

There was a layer of rubber-base material interposed between denture and bone, to simulate the action of mucosa and reduce or eliminate the impact disruption of lacquer.

* Stresscoat, Magnaflux Corp., Chicago 31, Ill.

Clinical experience led us to rubber-base material to use as a simulated mucosa between denture and bone. It certainly is not the same as normal mucosa, but it was thought that it was the closest substitute that could be used in the laboratory to provide the dampening action. In order to enhance this similarity, the material was formed in a pattern similar to the cross-sectional appearance of mucosa. Gurdjian and Lissner[6] did not think that the skin and muscles had much effect on the strain patterns. For example, the maxillary cushion was 1–1½ mm. thick over the ridge and gradually thickened to 3–4 mm. midway between the ridge and the midline, tapering then to 1 mm. over the midline. These were approximated thicknesses, as it was not considered necessary to

FIG. 1.—A "craze" sometimes occurs over the surface and can easily be distinguished from the patterns.

have them any more consistent than one would find in normal mucosa. Initially, ten thicknesses of rubber-dam material were used for this purpose but were later discarded because of the uniform thickness. It was thought that this uniformity might influence the strain patterns. Accordingly, the rubber-base method described above was used. A later comparison of photographs on six skulls showed no significant difference between the rubber dam and the rubber base. We continued the rubber-base method because it was simpler to manipulate during the tests. (Those patterns which occurred on the surface of the bone which is in contact with the denture were ignored because they could represent impact deformation. The authors feel, however, that this was not the case because patterns would often become apparent on the body and none immediately under the denture.)

The test stand was designed to hold the skull while the force was applied by means of

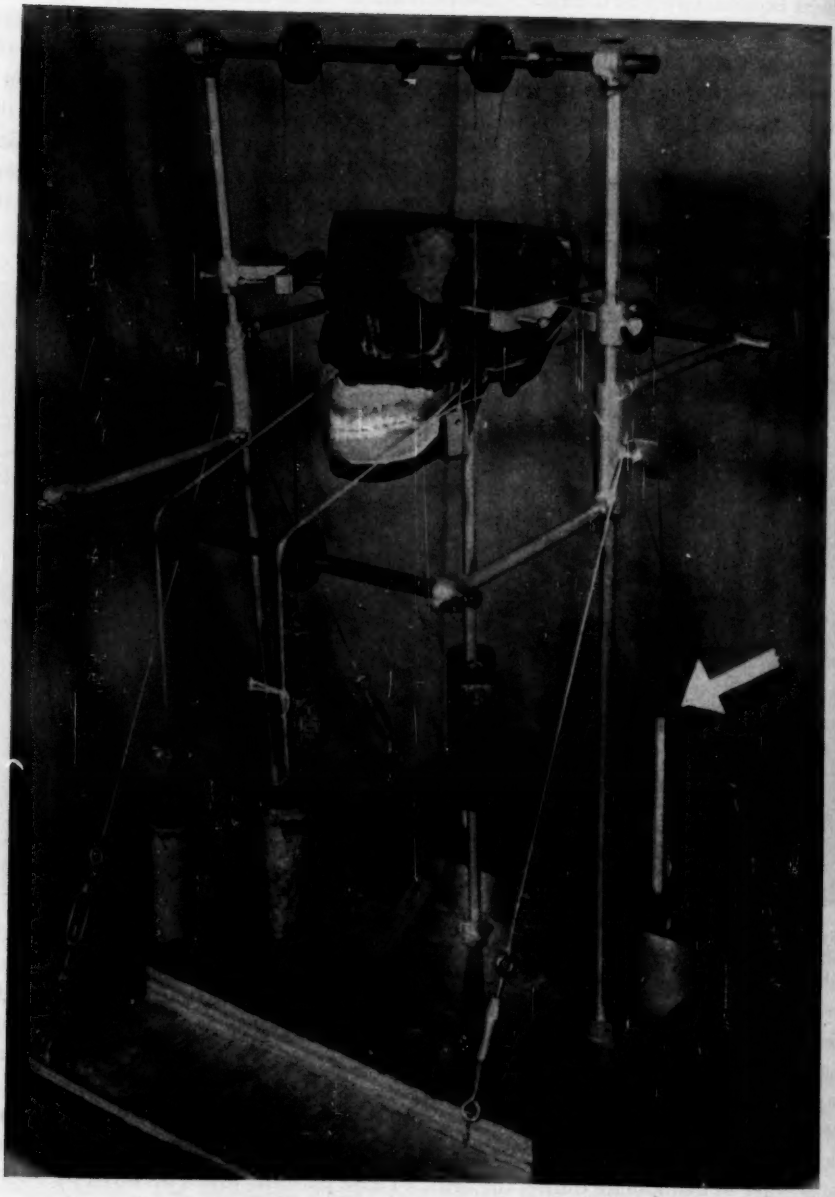

FIG. 2.—The testing stand. Arrow points to hydraulic dampers

kilogram weights operating over a pulley system in the general direction of masseter, temporal, and internal pterygoid muscles and then of the external pterygoid muscle.

The loading was done in a static manner, at first, in centric position, to determine what, if any, patterns occurred from the mere placing of the dentures. This was primarily a compressive force of 4 kg. When they occurred, they were generally limited to the basal seat. Then what may be termed "operating loads" were carried out. That is, additional kilogram weights were added slowly to the external pterygoid direction. This was accomplished by looping a cord around the neck of the condyle and passing it forward to a pulley and the weight hung on a loop at right angles to the direction of pull. This caused the lower jaw to move in eccentric positions. (No attempt was made to separate the types of forces. The intent was to simulate masticating activities and note the deformation patterns, if any occurred.) In order to prevent the eccentric movements from being violent jerks, hydraulic dampers were placed in a position to check the forward movement of the condyles.

Various types of temporomandibular articulations were considered in the early stages of the investigation. We were immediately faced with the impossibility of altering the integrity of the bony surface because this would affect the deformation patterns. In order to assure a smooth, slow temporomandibular articulation, we evolved the cord and hydraulic-damper arrangement described. It was necessary to lubricate the condyles with Vaseline to obtain smooth movement.

In the preliminary studies, condylar disks, fashioned to resemble cadaver disks, were glued to the condyles. These, made from rubber base, were also lubricated. Comparisons of strain patterns in six skulls with disks and the same six without the disks showed no essential difference. Therefore, in view of the added difficulty of making and gluing these, they were discontinued.

Gurdjian and Lissner[6] observed that "it can also be justifiably deduced that the effect of the skin and muscles on the strain pattern is negligible." Although these authors transmitted the force by means of a ball-peen hammer over a smaller area and we by means of the slow application of weights over a larger area, we feel that the results would be equally applicable in view of the qualitative results sought.

The resulting deformation patterns were identified by brushing an electrolyte (Stati-flux liquid)* over the surface and then wiping off the excess. This left the electrolyte in the cracks. An electrically charged white powder was blown over the surface, and the powder piled up on the electrolyte, making a white-on-black pattern on the skulls. This total procedure was carried out on the skull with anatomic tooth forms. The skull was then cleaned and the procedure repeated with the non-anatomic forms. Comparison of patterns occurring after (1) static loading, (2) anatomic forms in excursion, (3) static loading, and (4) non-anatomic forms in excursion showed the following results.

RESULTS

1. The results obtained supported the observations made in the preliminary investigations.[7] The anatomic tooth forms apparently caused more bone deformation over a larger area than did the non-anatomic forms (Figs. 3, 4).

2. Heavy bone structure seemed to be more resistant to deformation per unit force than average or light bone structure.

* Magnaflux Corp., Chicago 31, Ill.

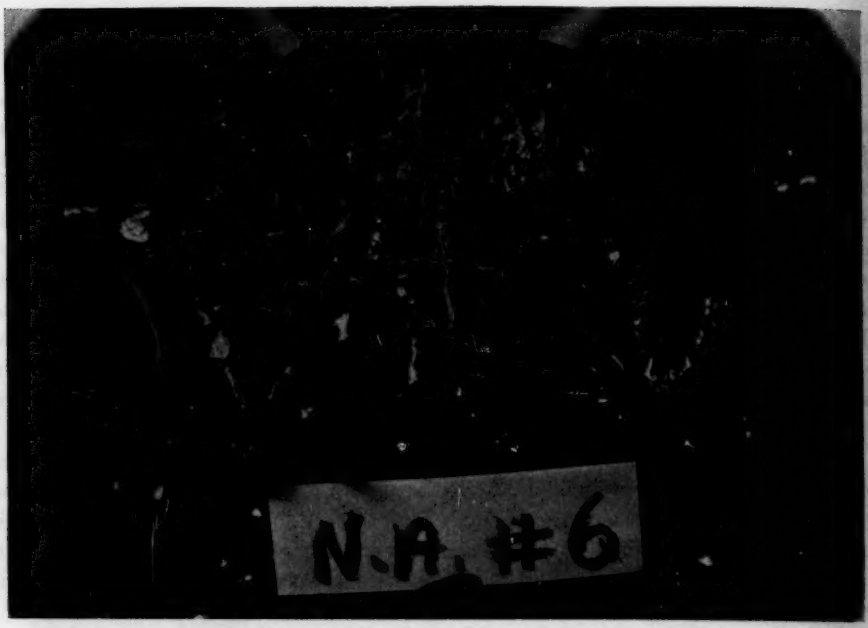

FIG. 3*A*.—Deformation patterns under non-anatomic teeth

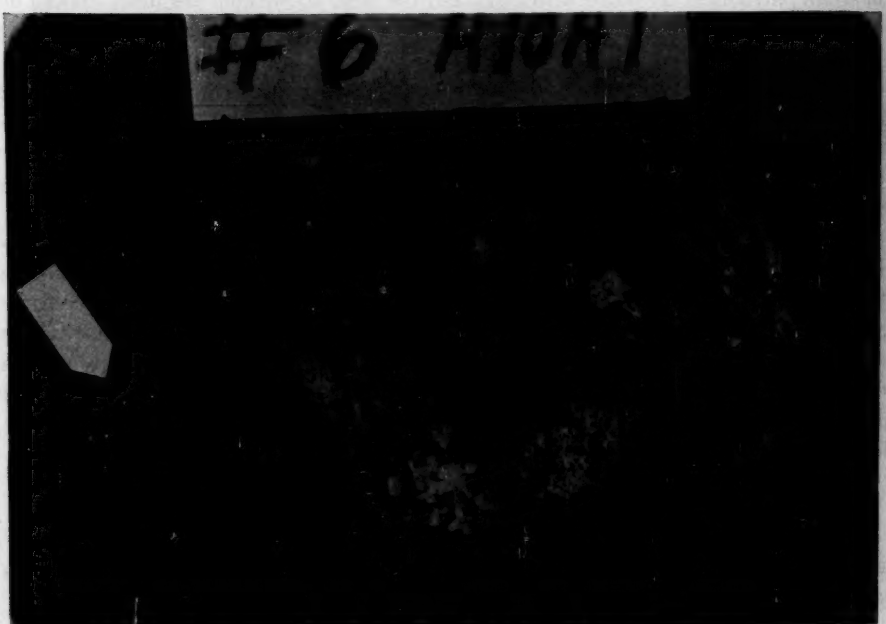

FIG. 3*B*.—Deformation pattern under anatomic teeth. Arrow points to increase of pattern over zygomatic ridge.

FIG. 3*C*.—Pattern under non-anatomic teeth on mandible

FIG. 3*D*.—Pattern under anatomic teeth

FIG. 4A.—Deformation patterns under non-anatomic teeth

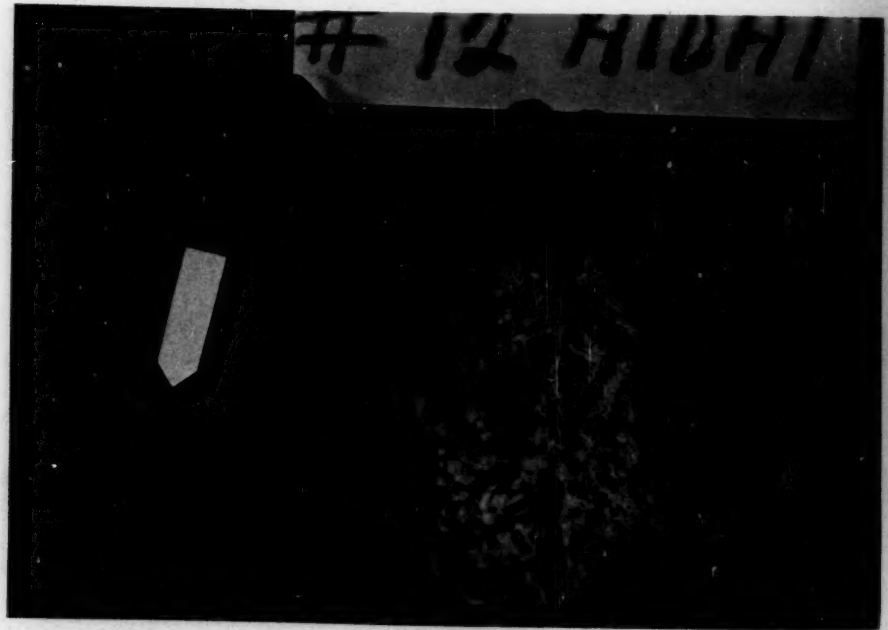

FIG. 4B.—Deformation pattern under anatomic teeth. Arrow points to increase of pattern over zygomatic ridge.

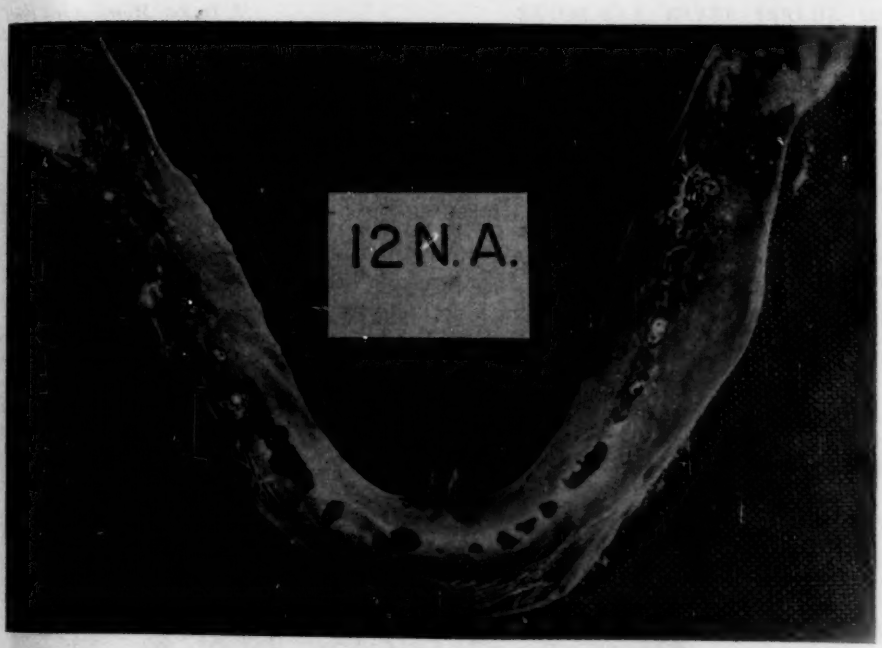

FIG. 4*C*.—Pattern under non-anatomic teeth on mandible

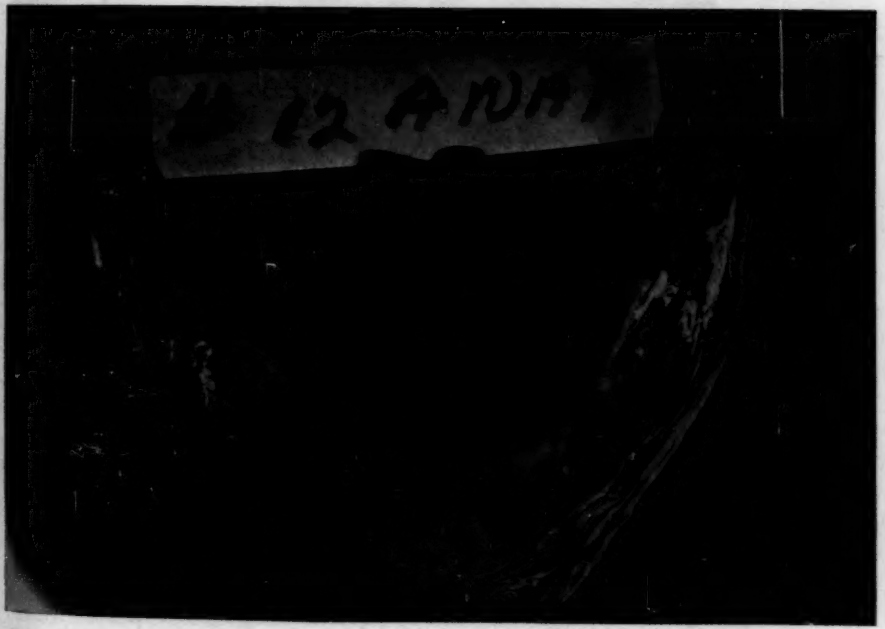

FIG. 4*D*.—Pattern under anatomic teeth

3. The deformation or stress patterns under dentures were not limited to the denture-bearing area but were rather widely distributed. Sometimes they might be seen on the zygomatic processes, medial wall of the orbit, and nasal bones (Figs. 5, 6).

4. The foramina seemed to be local areas where deformation occurred readily (Fig. 7).

DISCUSSION

Perhaps the most significant early Stresscoat work in biology was that carried on by Gurdjian and Lissner,[6] who wrote: "Consequently it is felt that a study of strain patterns based on dry skull preparations is accurate and represents conditions similar to

FIG. 5.—Deformation patterns in the medial wall of orbit

those obtained in the living organism." This, of course, makes these studies carried out in the laboratory of value to the clinician.

The lacquer will crack only as a result of tensile, not compressive, strain. This, however, is not so significant as it may seem because a force applied to the right side of a column will produce tensile strain on the left. Similarly, if a force is applied to the left side, it will produce tensile strains on the right. The resulting deformation patterns on the maxilla and mandible were never quite so "neat" as those in long bones and the skulls (not to mention those on metal parts).

Repeated procedures produced similar patterns, and, although these were not so regular and parallel in nature as seen in the work of other authors, there was indeed a parallelism which indicated the direction of the deformation. For example, cracks running from alveolus to lower border of mandible indicate a deformation caused by rami approaching each other (Fig. 8).

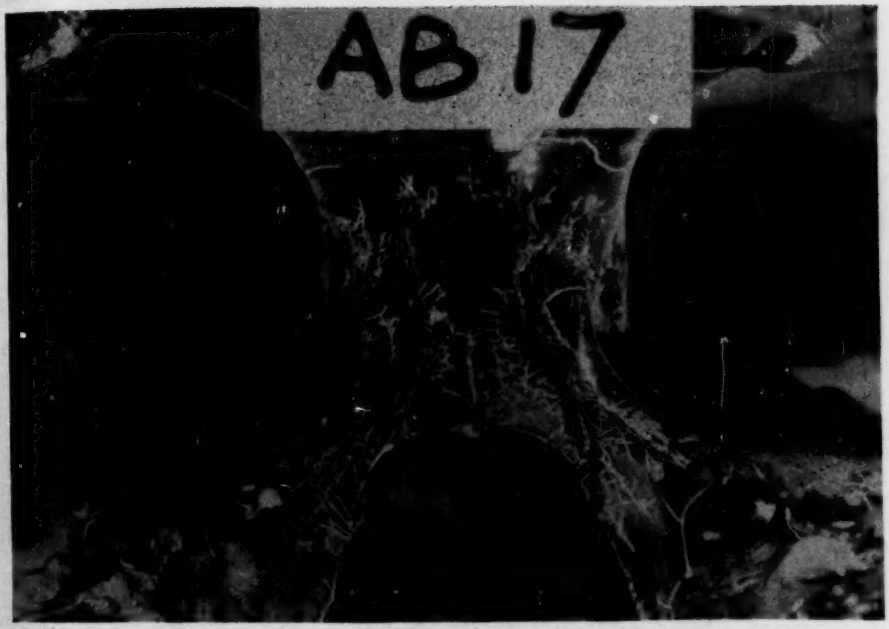

FIG. 6.—Deformation patterns on nasal bones

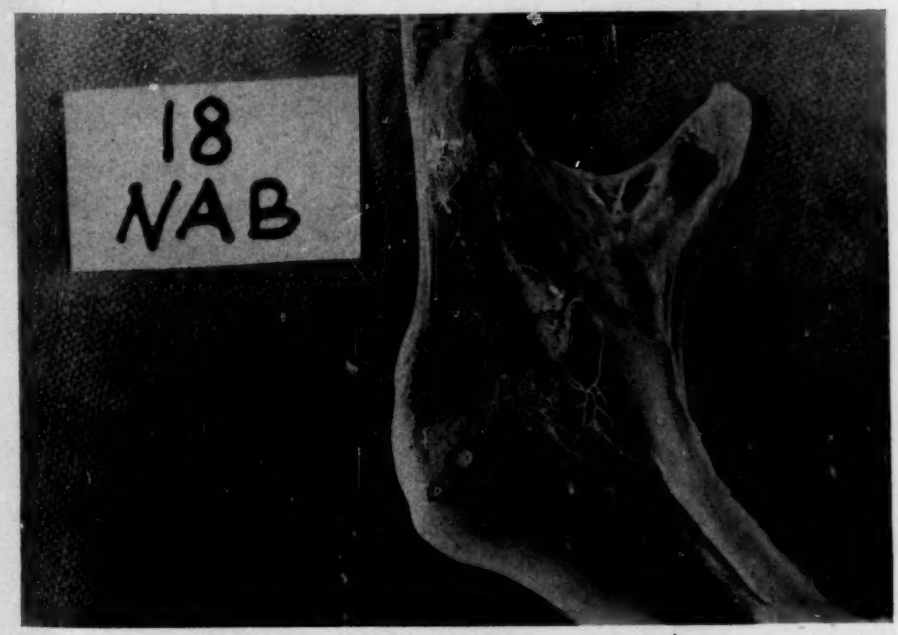

FIG. 7.—Deformation patterns around the foramen

Previous work on the mandible by Evans[8] and DuBrul and Sicher[9] was done on dentulous mandibles, and the presence of teeth perhaps modified the character of the deformation. In many mandibles the direction of cracks suggested that some deformation occurred as a result of the alveolar portion of the bones being moved over the body (Fig. 9). (The mental foramina present an obstacle to the continuation of these parallel cracks. Furthermore, in the anterior region the cracks are likely to shift to a vertical direction, as observed in dentulous mandibles.)

The structure of the bones and the buttress arrangement of the mandible, particularly, varied a great deal from one skull to another and made the picture rather more complicated than in long bones.

The skulls varied in weight from 324 to 1,009 gm. This was most readily apparent in the thickness and bulk of the mandibles, since no great difference in size was noted. The heavier bones were not deformed to the same degree as the lighter bones. In the latter, strain patterns readily occurred and were extensive.

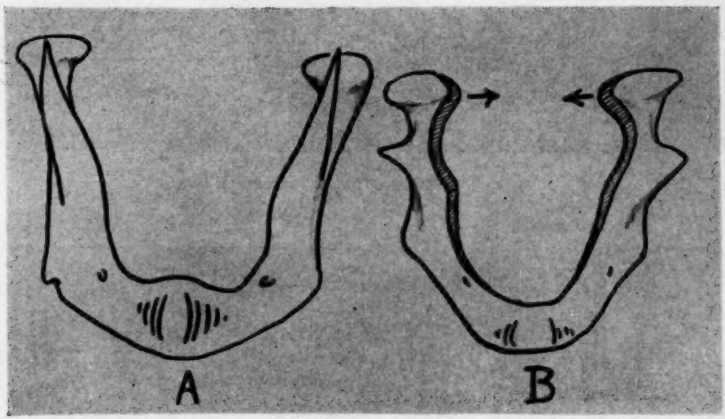

FIG. 8.—*A*, frontal view of mandible, showing vertical pattern near mid-line. *B*, the mandible from above showing how *A* was produced. Shaded area represents amount that condyles were brought toward each other to cause the vertical cracks.

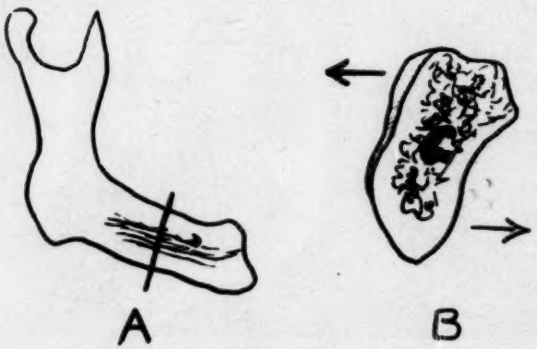

FIG. 9.—*A*, side view of mandible, showing typical pattern. Blank line represents area from which section *B* was taken. *B*, section showing how *A* patterns were produced. Shaded area represents the movement of alveolar portion over the body of the mandible.

In such heavy specimens, differences in strain patterns between anatomic and non-anatomic teeth were difficult to detect, as patterns tended to be minimal in any case. In the skulls of 500 gm. or less the differences between anatomic and non-anatomic patterns were most discernible. The latter were less extensive.

The patterns were reproducible in essence (although, of course, not in detail) and were compared by photograph and by note.

Six kilograms were chosen as the force to be applied because this was approximately half the force that average denture wearers could exert.[10, 11] It was felt that if deformation patterns occurred with this force, then they should occur at 12 kg.

The deformation patterns demonstrated at such places as the nasal bones and medial wall of the orbit, while not occurring in every skull, certainly pointed up the degree of deformation that may sometimes exist beneath complete dentures.

The authors encountered much more difficulty with the technique than has been experienced by other biological investigators. The humidity directly affected the lacquers, even though a correct lacquer was decided upon by psychrometer and thermometer. During the days of rain or other high humidity, it soon became apparent that tests should be postponed until "better weather" prevailed.

The question of *exactly how much* deformation occurs under various tooth forms can be answered by the use of strain gauges placed in those areas suggested by this study, which has afforded a gross estimate of difference.

SUMMARY

A method which utilizes a strain-sensitive lacquer to demonstrate deformation patterns in bone was applied to the study of these patterns in the mandible and maxilla. Dentures were made for each of eighteen edentulous skulls.

They were duplicates in all respects except that one had non-anatomic occlusal forms and the other had anatomic forms. These skulls with dentures were mounted on a testing platform and 6-kg. loads (simulating muscle pull) were applied while the mandible was moved into lateral and protrusive positions.

Results suggest that identical loads cause more deformation of bone beneath anatomic forms than beneath non-anatomic forms; that deformation extends to remote areas; that heavy bone structure resists deformation; and that foramina seem to be areas of increased deformation.

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Endotoxic Properties of Oral Bacteria as Revealed by the Local Shwartzman Reaction

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While a great deal of literature has accumulated concerning the endotoxic properties of Gram-negative bacteria from a variety of sources, little information is available on the occurrence of endotoxins in bacteria indigenous to the human oral cavity. A systematic investigation of some of the endotoxic manifestations of these bacteria may be of considerable importance in evaluating their role in periodontal disease. This study, representing one phase of this investigation, utilizes the dermal Shwartzman reaction[1] as an indicator of the endotoxic properties of certain oral bacteria.

MATERIALS AND METHODS

Three strains of anaerobic Gram-negative cocci (genus *Veillonella*), designated as V2, V3, and V4, were isolated from human saliva by means of the selective media of Rogosa *et al.*[2] The same medium, minus agar, vancomycin, basic fuchsin, and Tween 80, was employed for the collection of bacterial cells. Three strains of Gram-negative fusiform bacilli, designated as F1, F4, and F5, were isolated from human saliva on selective media as outlined by Omata and Disraely.[3] Cells were collected from the same medium, lacking agar, streptomycin, and crystal violet. An oral Gram-positive diphtheroid bacillus, D9, was grown in a casitone (Difco)–yeast extract medium previously described.[4] All these organisms were grown in 500-ml. portions of media in 1-liter Erlenmeyer flasks for 72 hours at 37° C. The cells were collected by centrifugation, washed in acetone, and dried *in vacuo* over calcium chloride. In addition, acetone-dried cells of an oral anaerobic streptococcus, JS9 (collected in a previous investigation),[5] were utilized as a source of Gram-positive bacteria.

Endotoxic solutions were prepared by tryptic digestion.[6] One hundred–milligram portions of the acetone-dried cells were incubated with 2.0 mg. of crystalline trypsin* in 10 ml. potassium phosphate buffer, pH 7.6, ionic strength 0.4, for 48 hours at 37° C., after which time the remaining bacterial debris was removed by centrifugation at 6000 rpm for 10 minutes in an angle centrifuge. The opalescent supernatants were decanted and heated at 80° C. for 1 hour, to inactivate the trypsin, and were filtered through a Selas O2 candle. The culture supernatants, collected after 72 hours' incubation, were filtered through a Selas O2 candle. All reagents were tested within a 24-hour period after collection.

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* Nutritional Biochemicals Corporation, Cleveland 28, Ohio.

The sterile soluble cellular products were tested for endotoxic activity by injecting them in 0.5 ml. volume intracutaneously into the shaved skin of New Zealand white rabbits weighing 1.5–2.0 kg. These animals were observed at various intervals from 2 to 24 hours after injection. Cellular extracts or cell-free culture filtrates were tested for their preparatory and provoking capabilities in the local Shwartzman reaction by injecting 0.5 ml. of the test substance intracutaneously, followed in 24 hours by the injection of a homologous or heterologous substance intravenously into the marginal ear vein. The area at the site of intracutaneous injection was observed at various time intervals up to 6 hours for the production of a hemorrhagic and necrotic lesion that

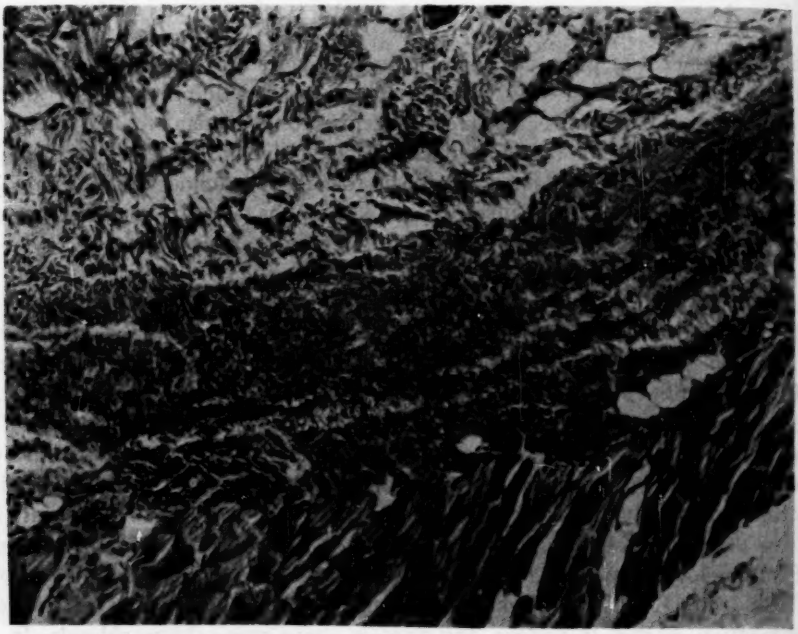

FIG. 1.—Tissue section from the inflammatory lesion produced by the intracutaneous injection of endotoxic products from fusiform bacilli (24 hours' duration; hematoxylin and eosin stain; ×105).

is typical of the dermal Shwartzman reaction. Selected animals were sacrificed, lesions were removed and fixed in 10 per cent formalin and imbedded in paraffin, and sections were stained with hematoxylin and eosin for histological examination.

RESULTS

The preparatory intracutaneous deposition of endotoxic products from fusiform bacilli or from Gram-negative cocci resulted in the formation of inflammatory lesions with a central ischemic area within 2 hours after injection. Such lesions, on histologic examination after 24 hours, proved to be sterile superficial areas of necrosis with scattered inflammatory cells (Fig. 1). After 48 hours the center of the lesions broke down into definite abscesses, which healed in approximately 7 days. In no case were hemorrhagic lesions observed after the preparatory injection. In contrast, soluble extracts

of the diphtheroid bacilli and the anaerobic streptococci produced only a transient inflammatory response with no histologic evidence of abscess formation after 24 or 48 hours.

The classical picture of the dermal Shwartzman reaction, characterized by extensive hemorrhage, edema, and necrosis with thrombosis and disruption of blood vessels, was produced with the endotoxic products from all strains of fusiform bacilli and *Veillonella* tested (Figs. 2–5; Table 1). The hemorrhage and necrosis thus produced had spread beyond the limits of the lesion caused by the preparatory injection alone. Cell-free culture filtrates from the *Veillonella*, V2, were incapable of preparing dermal sites for

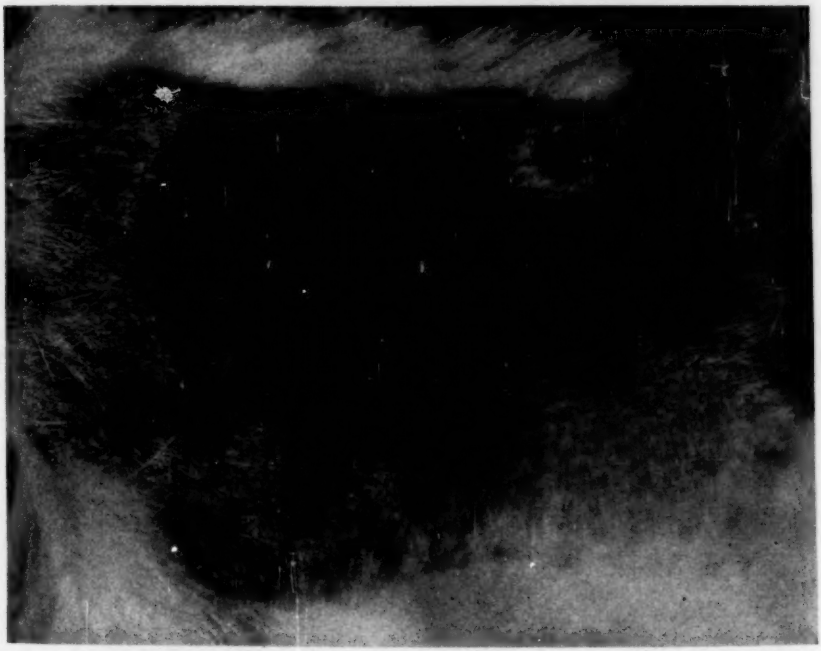

FIG. 2.—Hemorrhagic necrosis of dermal Shwartzman reaction in the rabbit produced by the preparatory and provoking injections of *Veillonella* endotoxic products (6 hours' duration). Original area of ischemia produced by the preparatory injection is visible.

this reaction in rabbits under conditions in which bacterial endotoxin was active. Interestingly enough, soluble products extracted with trypsin from the two strains of Gram-positive bacteria (anaerobic streptococci, diphtheroid bacilli) failed to elicit localized Shwartzman reactions in rabbits.

DISCUSSION

To investigate the possibility that bacterial endotoxins might play a role in the pathogenesis of periodontal disease, soluble cellular products of oral bacteria were prepared by tryptic digestion and examined for their capacity to elicit the localized Shwartzman reaction. Consistently positive results were obtained with endotoxic products from the strains of *Veillonella* and fusiform bacilli employed. The fact that 72-

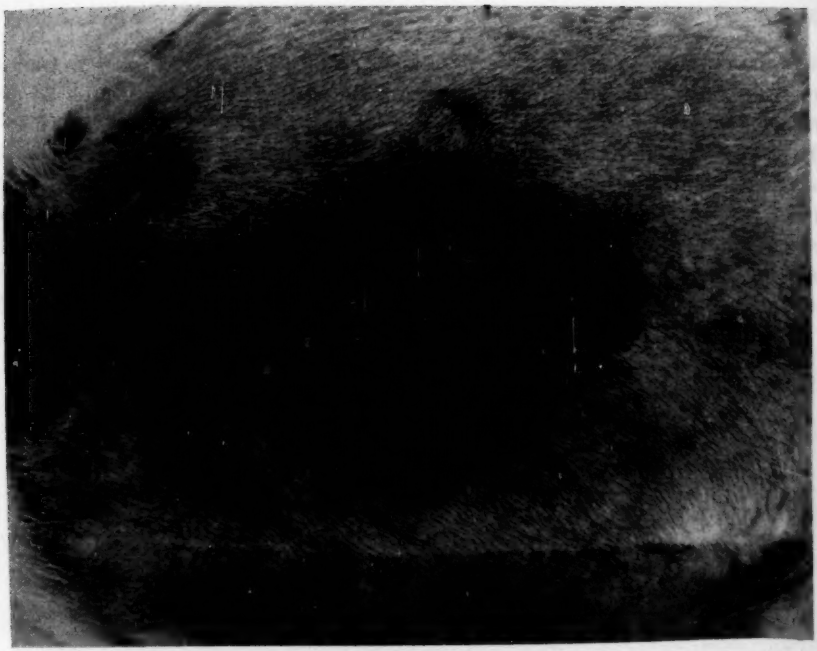

FIG. 3.—Hemorrhagic necrosis of dermal Shwartzman reaction in the rabbit produced by the preparatory and provoking injections of endotoxic products from fusiform bacilli (6 hours' duration). Original area of ischemia produced by the preparatory injection is visible.

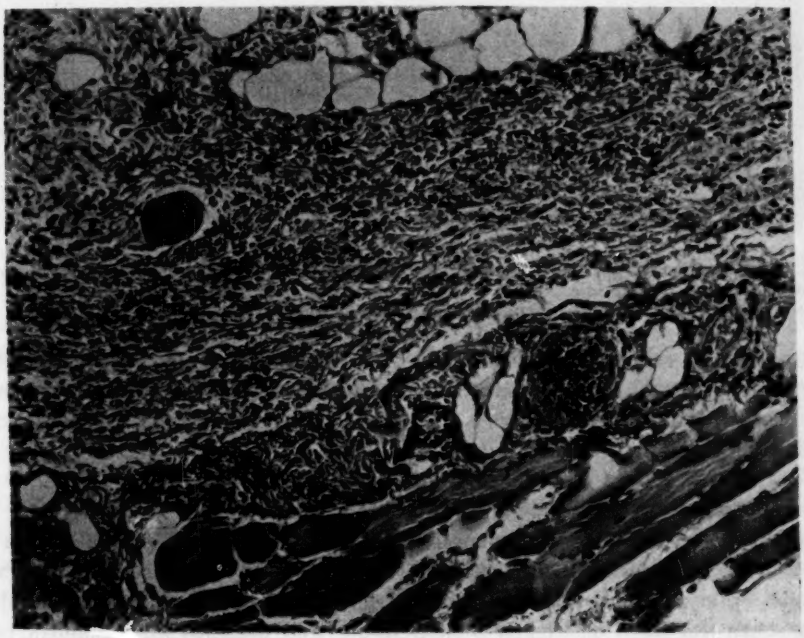

FIG. 4.—Tissue section from Fig. 2, demonstrating the massive leukocytic infiltration and engorgement of blood vessels with polymorphonuclear leukocytes (hematoxylin and eosin stain; ×143).

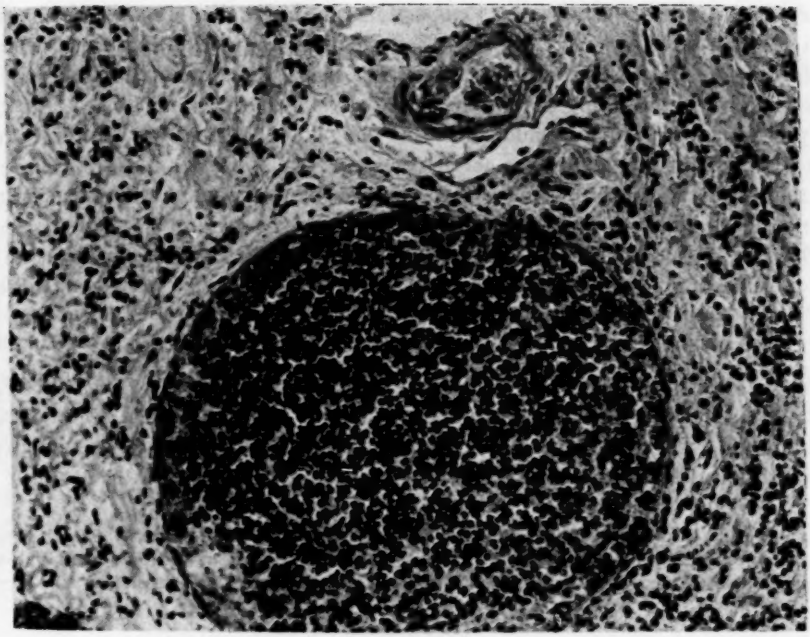

FIG. 5.—Tissue section from Fig. 3, showing, the engorgement of a large vessel with polymorphonuclear leukocytes (hematoxylin and eosin; ×210).

TABLE 1

LOCAL SHWARTZMAN REACTION WITH ENDOTOXINS FROM ORAL BACTERIA

Strain	Intracutaneous Preparing Dose (0.5 Ml.)	Intravenous Provoking Dose (1.0 Ml.)	No. Rabbits	No. Positive Reactions*
Veillonella, V2	Cell extract†	Cell extract	2	2
Veillonella, V2	Cell extract	Cell-free culture filtrate‡	2	2
Veillonella, V2	Cell-free culture filtrate	Cell-free culture filtrate	2	0
Veillonella, V2	Cell-free culture filtrate	Cell extract	2	0
Veillonella, V3	Cell extract	Cell extract	2	1
Veillonella, V4	Cell extract	Cell extract	2	2
Fusiform, F1	Cell extract	Cell extract	3	2
Fusiform, F4	Cell extract	Cell extract	3	3
Fusiform, F5	Cell extract	Cell extract	2	2
Diphtheroid, D9	Cell extract	Cell extract	2	0
An. strept., JS9	Cell extract	Cell extract	2	0

* Hemorrhage and necrosis, as in Figs. 2 and 3.

† Soluble products of tryptic digestion. Nitrogen values ranged from 1.5 to 2.5 mg/ml in each of the extracts employed.

‡ 72-hour cell-free culture filtrate.

hour cell-free culture filtrates of a *Veillonella* strain failed to prepare for the Shwartzman reaction probably indicates that these organisms do not autolyze in culture releasing their cellular endotoxins. On the other hand, these cell-free culture filtrates were effective in provoking the Shwartzman reaction after the animal had been given the preparatory injection of endotoxin. This finding is consistent with the observation that a number of substances can act as provoking agents in this reaction.[1] That this reaction cannot be elicited by all bacteria indigenous to the oral cavity was shown by the negative results with two strains of Gram-positive bacteria.

The finding that certain oral bacteria possess endotoxic properties may be an important clue in the search for factors related to the etiology of periodontal disease.

SUMMARY

Tryptic digestion of acetone-dried Gram-negative oral bacteria (*Veillonella, Fusobacterium*) released soluble endotoxins, which elicited hemorrhagic and necrotic lesions in the rabbit (Shwartzman reaction). Oral diphtheroid bacilli and anaerobic streptococci did not yield Shwartzman-reactive endotoxins after similar treatment.

The author wishes to express thanks to Dr. Harold Stanley for preparation of the histological specimens, to Mrs. Elizabeth Fath for the nitrogen determinations on the preparations of endotoxic products employed in these experiments, and to Mrs. Elizabeth Goodsell for technical assistance.

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Marginal Penetration of Dental Restorations by Different Radioactive Isotopes

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The degree of marginal seal achieved with dental filling materials has concerned the operative dentist for some time, and a variety of techniques have been utilized to study this problem.[1-3] More recently tracer solutions of dye[4] or radioactive calcium chloride[5,6] have been employed to detect the degree to which the margins of restorations were penetrated. A recent study used a combination of crystal violet dye with radioactive sodium iodide (I^{131}) as a tracer solution, to compare the influence of molecular size on the degree and path of penetration.[7] Previous studies were based on the use of only one radioactive tracer. The present study employed a variety of isotopes to determine whether ionic charge and chemical reactivity influenced the degree of marginal penetration.

TABLE 1

FILLING MATERIALS USED

Material	No. Teeth	Material	No. Teeth
Silver amalgam	21	Red-copper cement	4
Cast gold inlay	3	Copper amalgam	5
Gold foil	6	Zinc oxide–eugenol	17
Acrylic resin	16	Zinc phosphate cement	19
Silicate cement	17	Temporary stopping	4

MATERIALS AND METHODS

This study utilized 147 freshly extracted human teeth from male patients with an average age of forty-five years. The teeth were placed in a normal saline solution with 1 per cent glycerin and stored at 44° F. (7° C.) immediately after extraction. They were kept in this solution no longer than 6 hours before being used. The teeth were obtained from full-mouth extractions prior to full denture construction, so that different dental filling materials (Table 1) could be studied in teeth from the same patient. Complete records were kept of each patient, including preoperative full-mouth roentgenographs.

Open preparations containing no fillings were used as controls and for comparison (35 teeth).

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Isotopes used.—The radioactive isotopes used were S^{35} as $Na_2S^{35}O_4$, P^{32} as $Na_3P^{32}O_4$, Na^{22} as $Na^{22}Cl$, Rb^{86} as $Rb^{86}Cl_2$, and Ca^{45} as $Ca^{45}Cl_2$ (Table 2).

The concentrated radioactive isotope solution was diluted with distilled water to the specific activity required (Table 2, fifth col.). The teeth were placed in individual tubes, each containing 2 ml. of the radioactive tracer solution (pH 6.0–5.2) with the crowns downward to insure complete coverage during the 24-hour immersion period at room temperature. In order to determine whether the pH of the isotope solution affected the uptake and penetration, a series of isotope solutions were used at pH 5, 6, and 7. There was no significant difference in the findings at these pH levels.

Cavity preparation.—A Class V box-type cavity preparation was made in each tooth. Most preparations were made on the buccal or labial surface of the tooth. A few were made on the lingual surface when this surface presented the larger area of clinically sound enamel. Occasionally, preparations were made on both the lingual and the buccal

TABLE 2

CHARACTERISTICS OF ISOTOPES USED

Symbols and Mass No.	Half-Life	Elect. Charge	Beta Energy (Mev)*	Specific Activity (μc/ml)	Approx. Autograph Time (Hr.)	No. Teeth Used
$S^{35}O_4$	87 days	Negative	0.167	20	48	19
$P^{32}O_4$	14 days	Negative	1.71	20	1/2	22
Na^{22}	2.6 years	Positive	0.58	10–15	6	62
Rb^{86}	19 days	Positive	1.56	30	4	14
Ca^{45}	163 days	Positive	0.254	20	30	6
Total						147

* Million electron volts—the energy of emissions.

surfaces and filled with different materials for more direct comparison of marginal penetration.

Class V (cervical) box-type cavity preparations were used to reduce the many variables inherent in occlusal cavity preparations. It was found that marginal integrity was difficult to achieve with occlusal preparations because of pits and fissures and that the enamel was very often damaged by plaques and incipient decalcifications. Furthermore, it was found, during many preliminary trials, that penetration of the isotope into the dentin underlying the restoration was dependent upon the nature of the underlying dentin (i.e., its sclerosis and translucent zones), as well as on the amount of secondary dentin in the subjacent pulp. For these reasons, Class V cavity preparations were used in preference to occlusal restorations. Teeth were selected for having no cervical lesions in the enamel. This greatly reduced the variability of marginal penetration, as seen in the autoradiograph, by virtue of the smooth enamel margins and general absence of sclerotic and secondary dentin under the preparation.

Autoradiographic methods.—Two longitudinal cuts were made through the center of each filling to produce a planoparallel center slab slightly less than 1 mm. thick. The cut surfaces were then polished manually on silicon carbide abrasive paper No. 220A and washed thoroughly in distilled water to remove adherent radioactive particles.

The finished tooth slabs were taken to the dark room and placed between two bare intraoral X-ray films (Minimax extra-fast double coated), inserted between two plastic blocks (1¾ × 3 inches) and tightened by bolts at each end. This pressed the tooth section tightly against the films, thus increasing the sharpness and detail of the final autoradiograph. It was important for the tooth slab to be of equal thickness, so that the pressure exerted was equal on the flat surfaces of the specimen.

The specimens were placed in light-tight boxes and exposed for varying periods of time, from 1 to 24 hours. The exposure times were determined empirically. The films were then developed as in ordinary radiography according to the manufacturer's instruction.

Autoradiographs of each specimen were prepared to provide at least one slightly underexposed and one slightly overexposed, as well as a series of "correctly" exposed films. This was necessary for a critical evaluation of the depth of penetration of the isotope. In the figures, *0* = no marginal penetration; *1* = superficial penetration of isotope (generally through the cervical margin only); *2* = penetration to, but not including, the floor of the cavity; *3* = penetration all around the filling, including the floor of the cavity; *4* = diffusion of isotope into the dentin; and *5* = penetration of isotope into the pulp chamber. The illustrations were printed at five times magnification.

RESULTS

Unfilled cavities.—Four of the isotopes ($S^{35}O_4$, Na^{22}, Rb^{86}, and Ca^{45}) penetrated through the freshly cut dentin of the open cavities to the pulp chamber within 24 hours. However, $P^{32}O_4$ did not reach the pulp chamber within this time period. Figures 1 and 2 compare the average depth of penetration of the various isotopes, the number of teeth used, and the variations in depth of penetration which occurred. Figures 3, 4, 5, and 6 illustrate the autoradiographs obtained with these isotopes.

Silver-amalgam restorations.—Silver-amalgam restorations immersed in S^{35}-labeled sulfate, Ca^{45}, and Rb^{86} solutions showed penetration of these isotopes around the filling margins, through the dentin to the pulp chamber (Figs. 3 and 4). Radiosodium (Na^{22}) also penetrated deeply, to the pulp chamber in some specimens and almost to the pulp in others (Fig. 4, *top*). However, the penetration of P^{32}-labeled phosphate was very superficial, reaching only partway along the cervical and incisal walls (Fig. 3, *bottom*).

Silicate-cement restorations.—Teeth restored with silicate cement and immersed in Na^{22} solution showed marginal penetration of this isotope through the dentin and, in most cases, to the pulp chamber (Fig. 5, *bottom*). However, the teeth immersed in S^{35}-labeled sulfate showed penetration around the filling to the floor of the cavity but not into the underlying dentin (Fig. 5, *top*), while those immersed in Ca^{45} showed even less penetration, the isotope penetrating only along the cavity walls but not to the floor of the cavity (Fig. 5, *middle*).

Acrylic-resin restorations.—The autoradiographs of the teeth restored with acrylic resin and immersed in Na^{22} and Ca^{45} solutions showed penetration by these isotopes through the filling margins and into the underlying dentin to the pulp chamber (Fig. 5, *right*). Teeth immersed in Rb^{86} solution showed marginal penetration into the dentin, but not reaching the pulp chamber. Autoradiographs of the teeth immersed in $S^{35}O_4$ solution showed penetration around the fillings up to and including the floor of the cavity, but not into the underlying dentin (Fig. 5, *top*).

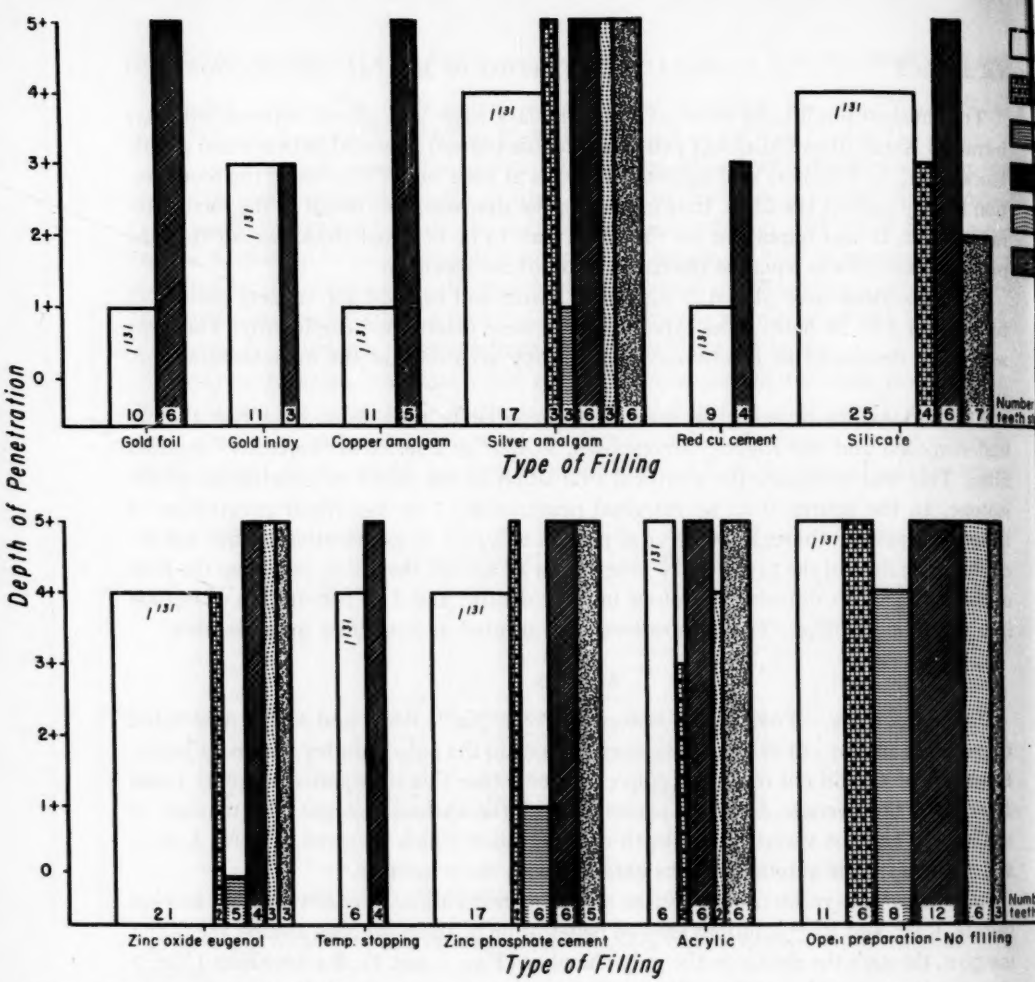

FIG. 1.—Histogram showing the average depth of isotope penetration around filling margins. 5+ indicates penetra the pulp chamber.

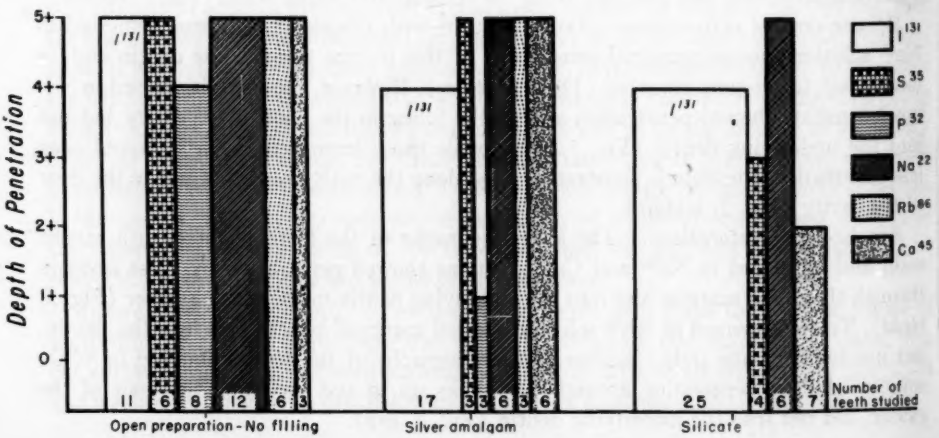

FIG. 2.—Enlargement of Fig. 1, comparing depth of isotope penetration around silver-amalgam and silicate-cement restorations with unfilled cavities.

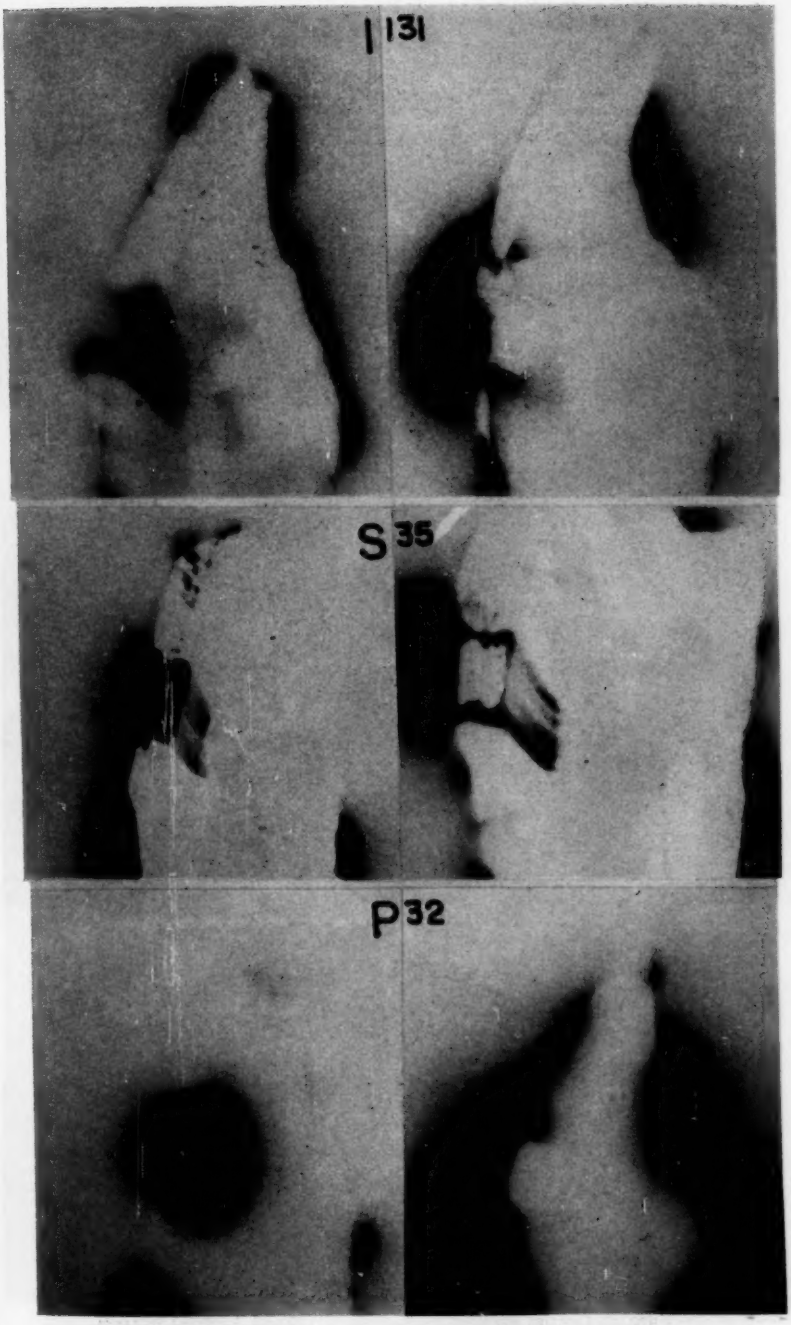

Fig. 3.—Autoradiographs of tooth sections, showing penetration of radioiodide, radiosulfate, and radiophosphate through the dentin of unfilled cavities and through the margins of cavities filled with silver amalgam. Mag. ×5.

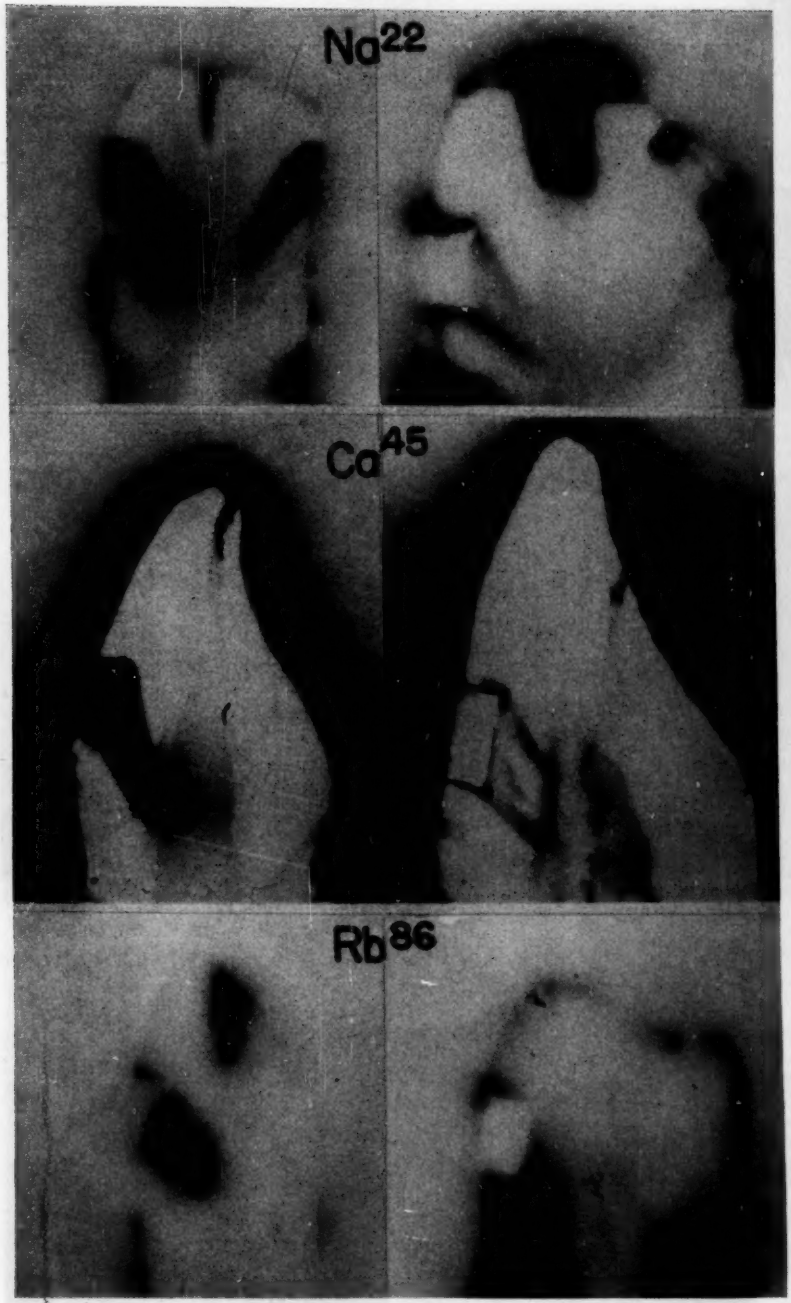

Fig. 4.—Autoradiographs of tooth sections, showing penetration of radiosodium, radiocalcium, and radiorubidium through the dentin of unfilled cavities and through the margins of cavities filled with silver amalgam. Mag. ×5.

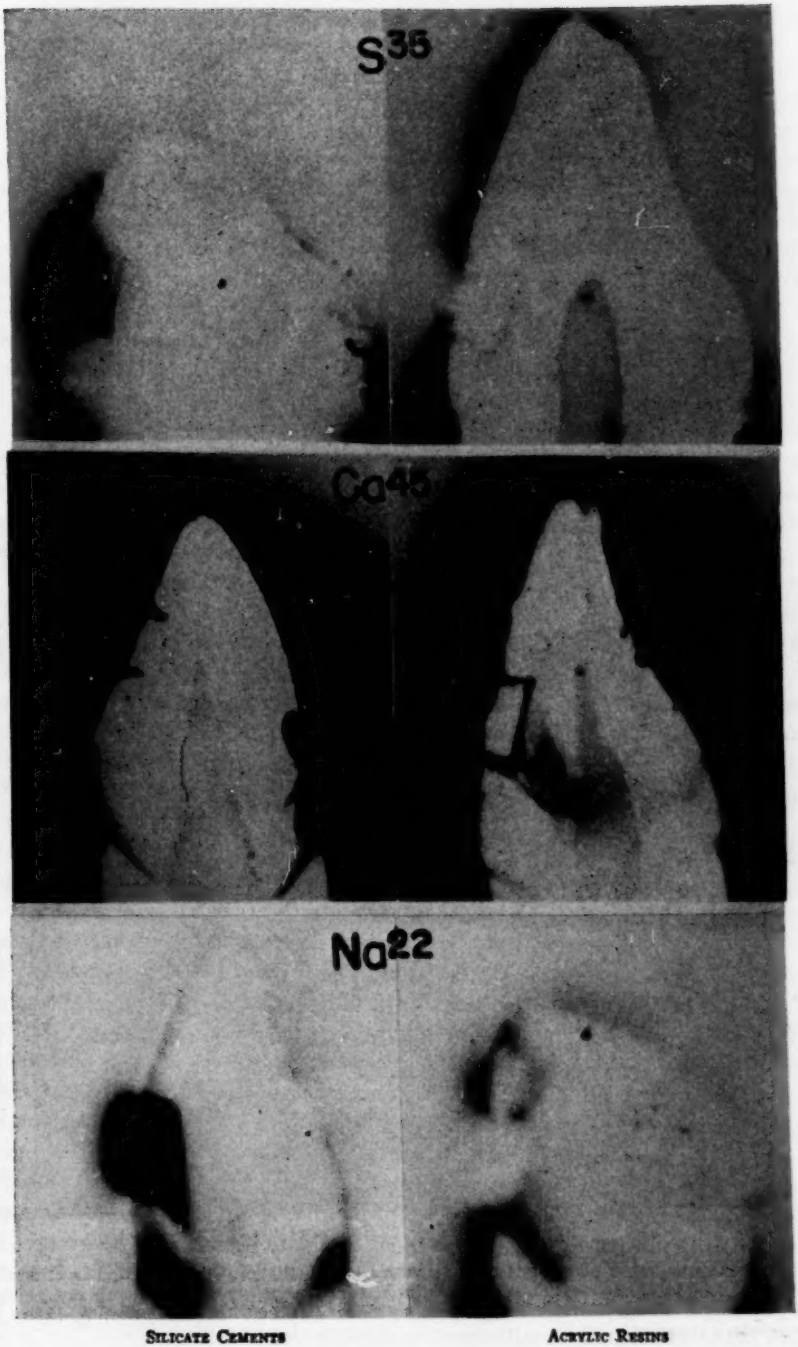

FIG. 5.—Autoradiographs of sections of teeth with Class V fillings of silicate cements and acrylic resins immersed in radiosulfate, radiocalcium, and radiosodium for 24 hours. Mag. ×5.

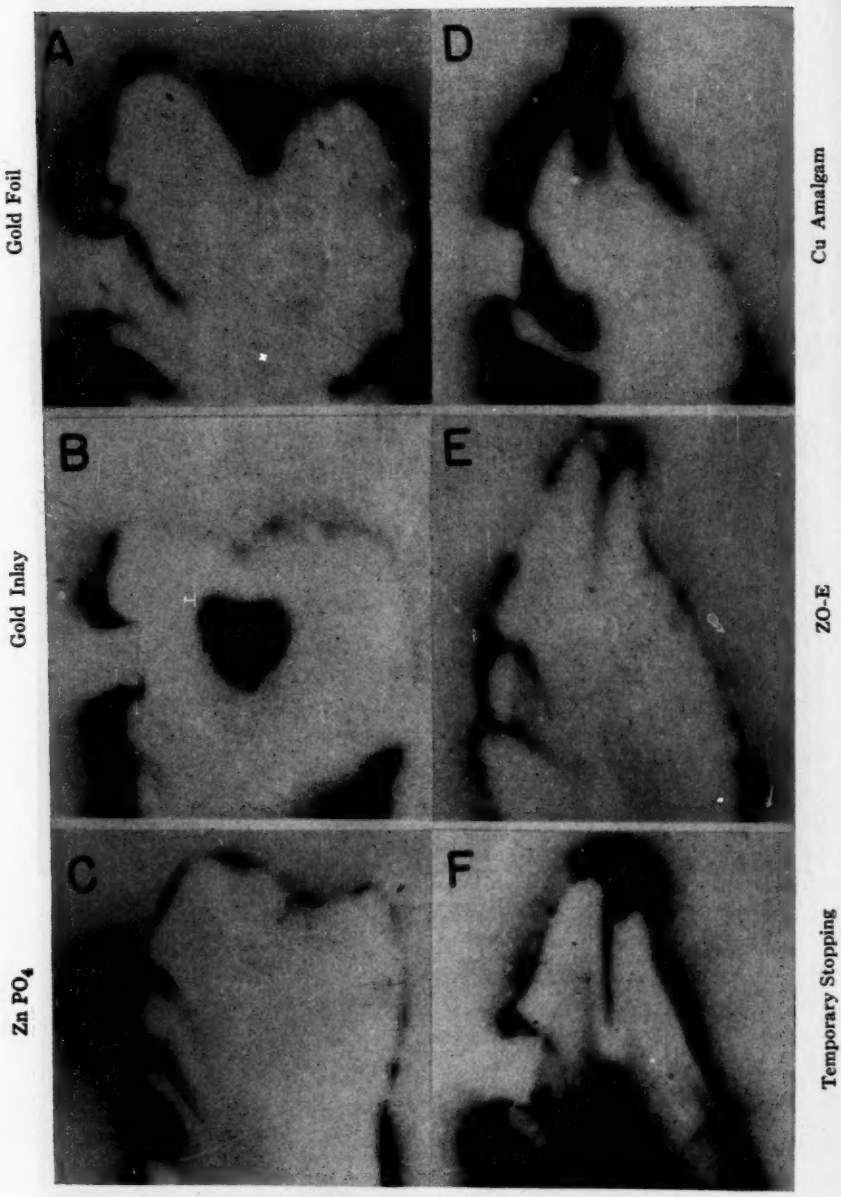

FIG. 6.—Autoradiographs of sections of teeth with Class V fillings after immersion in radiosodium for 24 hours. *A*, gold-foil restoration; *B*, gold-inlay restorations; *C*, cavity filled with zinc phosphate cement; *D*, copper-amalgam restoration; *E*, cavity filled with zinc oxide–eugenol; *F*, cavity filled with temporary stopping.

Zinc oxide–eugenol restorations.—Radiosodium (Na^{22}) penetrated the margins of zinc oxide–eugenol restorations and passed through the dentin to the pulp chamber (Fig. 6, *E*). Three additional teeth filled with zinc oxide–eugenol to which 1 drop of a saturated solution of zinc acetate was added to hasten the set showed a reduced penetration of Na^{22} solution. The Na^{22} penetrated into the dentin but did not reach the pulp chamber. However, the teeth restored with fast-setting zinc oxide–eugenol (zinc acetate added) and immersed in Rb^{86} showed penetration of this isotope through the filling margins to the pulp chamber. Teeth restored with plain zinc oxide–eugenol and immersed in Ca^{45} also showed marginal penetration through the dentin to the pulp chamber, while those immersed in $S^{35}O_4$ showed penetration of this isotope into the dentin only. Finally, the teeth immersed in $P^{32}O_4$ showed no marginal penetration by this isotope (Fig. 1, *bottom left*).

This series brings out the fact that a comparison of marginal penetration among the different filling materials depends greatly upon the tracer used. Dye studies showed less marginal penetration with zinc oxide–eugenol than with acrylic resin, silicate cement, red copper cement, or zinc phosphate cement and only very little more than with the metallic fillings.[7] The same conclusions might be reached when $P^{32}O_4$ is used as the tracer. However, the results were quite different when Na^{22}, Ca^{45}, Rb^{86}, or $S^{35}O_4$ was used. The latter group of tracers indicated that the margins of zinc oxide–eugenol restorations were as penetrable to these ions as were the margins of acrylic-resin restorations.

Zinc phosphate cement restorations.—Teeth restored with zinc phosphate cement and immersed in Na^{22} and in $S^{35}O_4$ solutions showed penetration by these isotopes through the filling margins and the dentin to the pulp chamber (Fig. 6, *C*). Penetration by Ca^{45} was limited along the incisal walls, but at the cervical margins the Ca^{45} penetrated deeply through the dentin to the pulp chamber, as did Na^{22} and S^{35} (Fig. 1). Penetration by P^{32} was again very superficial and in marked contrast with the deep penetration observed with the other isotopes (Fig. 1).

The zinc phosphate cement restorations thus showed very poor marginal sealing, as poor as the acrylic-resin and zinc oxide–eugenol fillings.

Gold-foil and copper-amalgam restorations.—Teeth restored with gold foil and with copper amalgam showed penetration of Na^{22} solution around the margins of the restorations, through the dentin, and into the pulp chamber (Fig. 6, *A* and *D*). This was in marked contrast with the good marginal seal reported previously with these materials using I^{131} as the radioactive tracer solution (Fig. 1).[7]

Temporary-stopping restorations.—The four teeth restored with temporary stopping and immersed in Na^{22} solution showed marginal penetration through the dentin to the pulp chamber (Fig. 6, *F*). One filling was sealed in the cavity with a hot instrument, another with chloroform, another with oil of eucalyptus, and the fourth was placed in a cavity saturated with chloroform. The depth of marginal penetration was the same in each case, i.e., to the pulp chamber.

Red-copper-cement restorations.—Teeth restored with red copper cement and immersed in Na^{22} solution showed only superficial penetration of the isotope at the incisal margins. However, the cervical margins showed a variation in the depth of penetration from superficial in some specimens to deep penetration through the dentin to the pulp chamber in others.

Gold-inlay restorations.—The three teeth restored with gold inlays and immersed in Na^{22} solution showed much less marginal penetration than did any of the other fillings studied (Fig. 6, *B*). The Na^{22} penetrated both margins but stopped at the floor of the cavity.

DISCUSSION

Surface adsorption of isotopes and penetration of fillings.—In general, the metallic fillings showed a marked adsorption of the negatively charged isotopes I^{131}, $S^{35}O_4$, and $P^{32}O_4$, the positively charged ions (Na^{22} and Rb^{86}) showing little or no surface adsorption (Figs. 3, 4, and 6). Silver amalgam, in addition, showed a penetration of radiosulfate and radioiodide into its substance (Fig. 3). Radiocalcium also adsorbed strongly on the surface of the silver amalgam (Fig. 4, *center*).

The cements (silicate cement, zinc phosphate cement, red-copper cement, and zinc oxide–eugenol) showed marked surface adsorption and filling penetration by all the isotopes used, but to greatly varying degrees. In contrast, the acrylic resins and the temporary stopping showed no autoradiographic evidence of surface adsorption and no penetration into its substance.

Because of the small size of the fillings in the natural teeth and the fact that they were placed within tooth tissues which also adsorbed the radioions, a more detailed analysis of surface adsorption and filling penetration by various ions was difficult. Therefore, a large plug of each filling material was formed in an acrylic-resin dye and then placed in the isotope solutions. The adsorption of ions on the surface and their penetration into the substance of the filling material could then be studied with much greater clarity and accuracy. The results of this study will be reported in a subsequent paper.

Comparison of isotopes and autoradiographs.—One of the purposes of this investigation was to determine which isotope would be most suitable for the study of marginal leakage around fillings and subsequent dentin penetration. There was little doubt that S^{35}-labeled sulfate and Ca^{45} showed selective and deep penetration into marginal defects and produced the clearest and sharpest autoradiographs (Figs. 3–5).

The isotope which least effectively penetrated into the cut dentin of the open cavities and around the fillings was P^{32}-labeled phosphate, a natural component of calcified tooth structure. It also produced a very diffuse autoradiograph (Fig. 3). Both factors limit its usefulness in studies of marginal leakage.

Na^{22} solution penetrated deeply and diffusely through minor defects in the tooth structure and even through intact structures. However, these autoradiographs also lacked contrast and detail and failed to demarcate clearly the pathway of marginal penetration (Figs. 4–6).

Rb^{86}, though capable of indicating the path of marginal penetration, did not produce survey autoradiographs of sufficient detail to be of value (Fig. 4). The sharpness of detail was superior to that obtained with P^{32}-labeled phosphate but inferior to that seen with Na^{22}.

The autoradiographs produced by I^{131} were not so sharp as those produced by $S^{35}O_4$ and Ca^{45} but were greatly superior to those produced by $p^{32}O_4$, Na^{22}, and Rb^{86}.

The autoradiographic technique.—The autoradiograph locates the position of the isotope by virtue of the effect of the beta particle on the silver grain. After development, a reduced grain of silver is found in the emulsion opposite the sites of radioactivity. According to Gross, Bogoroch, Nadler, and Leblond,[8] the resolution and density of the autoradiograph depend on "(*a*) the geometrical relationship of the radioactive source and overlying emulsion, (*b*) the energy and the intensity of the radiation and (*c*) the characteristics of the photographic emulsion." In this study factors *a* and *c* were constant, but the energy of the radiation *b* varied. The energy of the beta particle (its Mev) determines the length of track produced by the beta particle in the photographic emulsion and therefore its photographic efficiency. For example, P^{32}, with a high Mev (1.71) of its beta particle, produced a long track in the photographic emulsion, which affected silver grains at quite a distance and at various angles. The result was a diffuse picture due to the wide halo around each primary silver grain. S^{35}, with a low Mev (0.167), produced a short, straight track which activated only a few silver grains without excessive halation. The result was a sharp autoradiograph.[9]

It would be desirable to study in more exact detail the path of isotope penetration through intact and injured enamel, dentin tubules, and cavity margins. The coarse emulsion of the X-ray film does not permit sufficient enlargement for this purpose. In addition, the exact relation between the particle track in the emulsion and the underlying tooth structure is lost when the film is removed. To achieve a more detailed examination of the path of isotope penetration through tooth structures, it becomes necessary to use a strip-on film technique, whereby a very fine-grain emulsion is placed on the tooth section and remains there after development. This permits high-power microscopic location of the silver grains in relation to the tooth structure.[10] This technique is now being used for further study of this problem.

SUMMARY

This investigation was designed to compare the marginal penetration of most of the filling materials in common clinical use, employing a variety of radioactive isotopes under standardized conditions in vitro.

Class V cavities were prepared in 147 freshly extracted human teeth and were restored with either silver amalgam, cast gold inlay, gold foil, acrylic resin, silicate cement, red copper cement, copper amalgam, zinc oxide–eugenol, zinc phosphate cement, or temporary stopping. Unfilled cavities were used as controls.

The experimental teeth were immersed in the isotope solutions (10–30 μc/ml) for a 24-hour period. A series of different isotopes was used in this study rather than a single tracer, since preliminary studies showed that the charge on the ion and its chemical affiinity greatly influenced its adsorption on the surface of the filling material and on the tooth surface, as well as its penetrability through the margins of the restorations. The following radioactive compounds were selected on the basis of ionic charge and chemical activity: $Na_2S^{35}O_4$, $Na_3P^{32}O_4$, $Na^{22}Cl$, $Rb^{86}Cl_2$, and $Ca^{45}Cl_2$. Ground sections were prepared and multiple autoradiographs were obtained under standardized conditions.

All fillings showed some degree of marginal penetration to one or more of the tracers

used. A single order of marginal permeability could not be established, since the order of penetration varied somewhat with different isotopes. In general, the metallic restorations were superior to the non-metallic restorations under the conditions of this experiment. The behavior of the cement restorations was very variable, and the order of marginal penetration among silicate cement, zinc phosphate cement, and zinc oxide-eugenol could be reversed by the use of different isotopes. It is concluded that the ionic charge and chemical reactivity of the ion, as well as the physical and chemical nature of the restorative material, influence the depth of marginal penetration by the isotope.

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The Correlation of Lactobacillus *Count and Gamma Globulin Level of Human Saliva*

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The observation that strains of *Lactobacillus* failed to grow in vitro in the presence of human fraction II globulin[1] led us to consider the possibility that *Lactobacillus* counts per cubic centimeter of saliva might be a function of gamma globulin levels of saliva. Recently, Green demonstrated that pooled saliva from caries-susceptible individuals contained less gamma globulin-active saliva samples than saliva from caries immune individuals.[2] The objective of this investigation was to determine whether the gamma globulin level of saliva was in some way correlated with the *Lactobacillus* count obtained from such saliva.

MATERIALS AND METHODS

Five cubic centimeters of paraffin-stimulated saliva were collected from each of thirty-eight students 2 hours after breakfast. One cubic centimeter of saliva was used to inoculate Rogosa agar for culturing strains of *Lactobacillus*. Two cubic centimeters of saliva were used for total protein, alpha, beta, and gamma globulin determination, using the sodium sulfate method for precipitation, and were measured spectrophotometrically.

Two cubic centimeters were used for direct qualitation of gamma globulin. In this instance, rabbit antihuman gamma globulin serum bound to latex* was used for macroscopic determination of precipitation of saliva gamma globulin.

The method for *Lactobacillus* culture is well known and will not be described. The macroscopic precipitation test was carried out by centrifuging 1 cm^3 each of boiled and raw saliva. One drop of the supernatant was diluted in saline-glycine buffer: 1/1, 1/4, 1/8, on an appropriate slide. One drop of the antihuman gamma globulin rabbit serum was added to each of the dilutions and read after 2 minutes. The clumping of the globulin-bound latex made macroscopic determination of antibody reaction possible. In view of false-positive tests secured with boiled saliva, the results will not be reported. However, in all cases of 1/1 and 1/4 dilution there was a positive test for gamma globulin. Roughly approximated, this is something below 50 mg. per cent of gamma globulin.

The sodium sulfate determination of gamma globulin was carried out as follows: To a 0.5-ml. sample of the supernate of centrifuged saliva was added 9.5 ml. of 27.2

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per cent sodium sulfate solution. This was refrigerated for 3 hours and then centrifuged.

To the supernate was added 1 ml. bromosulfalein solution and 1 ml. of McIlvaine's buffer (pH 2.5) and then allowed to stand 30 minutes. This was centrifuged, the supernate was poured off, and the tube inverted and allowed to drain 3 minutes.

One milliliter of 10 per cent sodium hydroxide was added to dissolve the precipitate, and this was transferred quantitatively to a 25-ml. volumetric flask and diluted to volume.

TABLE 1

Lactobacillus COUNT, CARIES INDEX, AND GAMMA GLOBULIN LEVELS

Lactobacillus Count (0–5,000/Cc)	Globulin (Mg. Per Cent)	*Lactobacillus* Count (5,000+)	Globulin (Mg. Per Cent)
0	10.0	5,100	1.0
0	0.7	6,000	0.7
0	0.6	6,700	1.9
0	1.0	6,900	4.6
0	2.5	7,000	1.0
0	1.0	10,000	2.8
0	1.6	33,000	1.0
0	6.3	37,000	1.0
0	0.2	42,000	1.0
0	0.9	45,000	3.4
0	7.6	55,000	1.9
0	1.0	60,000	3.6
0	0.2	130,000	0.9
0	1.0	209,000	2.5
0	0.2		
0	1.0		
100	6.6		
100	2.5		
200	0.2		
300	2.8		
1,000	0.7		
1,700	2.5		
2,500	9.1		
4,200	1.0		
Total	61.2	Total	27.3
Average	2.55	Average	1.95

The per cent transmission was read on a Coleman spectrophotometer at 570 mμ, and the protein in the sample was determined from a standard graph.

RESULTS

The *Lactobacillus* counts ranged from 0 to 209,000. Such counts were separated into two groups. One group, caries-susceptible, included all counts above 5,000 per cubic centimeter; the other group, non-caries-susceptible, included counts below 5,000 per cubic centimeter. The gamma globulin determinations for each such counts were compared (Table 1).

The total gamma globulin in each group was summed and averaged. The quantity of gamma globulin in the caries-susceptible group was 2.55 mg. per cent. This represents a difference of 0.60 mg. per cent or 31.0 per cent.

Statistical treatment of values in Table 1 shows a small shift in the arithmetical mean (Table 2). The standard deviation of the means of gamma globulin, 2.55 and 1.95 in *Lactobacillus* counts of 0–5,000 and 5,000+, were 2.98 and 1.24, respectively.

The gamma globulin mean values were treated by the *t*-test: $t = 0.987$;* $S\bar{Y} =$ 0.403 mg. per cent.†

The significance of the *t*-test showed a chance probability of greater than 10 per cent.

* Probability. † Mean.

TABLE 2*

STATISTICAL VALUES OF *Lactobacillus* COUNTS AND GAMMA GLOBULIN LEVELS

Lactobacillus Counts	*S*		*N*		Fix.		$\bar{x}$		SD	
	LA	GG (Mg. Per Cent)	*LA*	GG	*LA*	GG	*LA*	GG (Mg. Per Cent)	*LA*	GG
0-200,000	.....	88.5	.....	38	.....	93	.....	2.45	.....	0.403
0-5,000	10,100	61.2	24	24	14,500	65	604.16	2.70	678.2	2.98
5,000+	652,700	27.3	14	14	640,000	31	45,714	2.21	547,723	1.24

* The letters in columns have the following meanings: *S* = sum of observations; *N* = number of observations; Fix. = product of frequency and observation; $\bar{x}$ = mean; and SD = standard deviation.

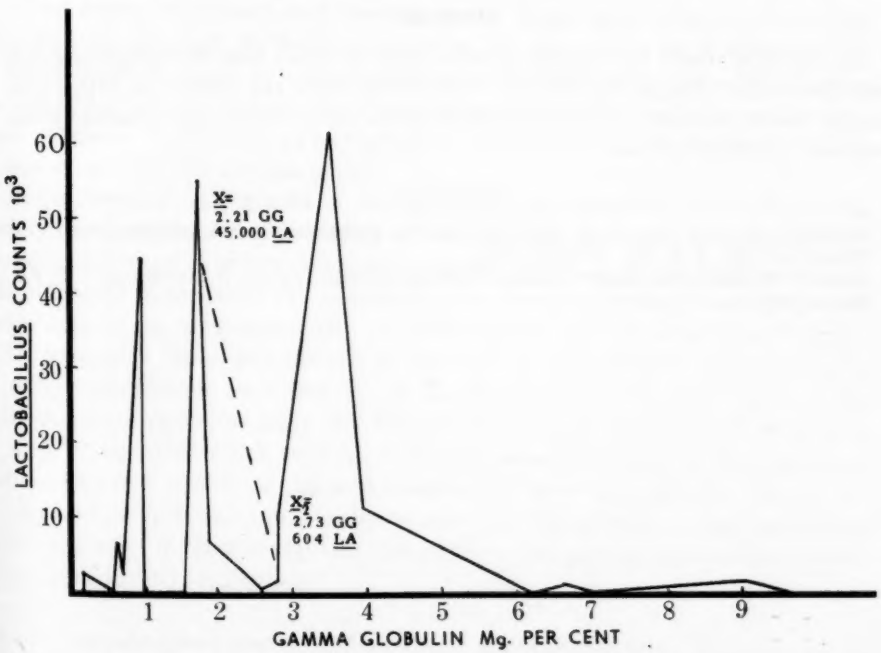

FIG. 1.—Co-ordinates of *Lactobacillus* counts and gamma globulin levels of thirty-eight subjects. Broken line joins mean value co-ordinates of gamma globulin and *Lactobacillus* counts. $X =$ above 5,000 *Lactobacillus* per cubic centimeter of saliva. $X_2 =$ below 5,000 *Lactobacillus* per cubic centimeter of saliva.

This implies that more than one out of ten of the gamma globulin values might be observed by chance alone.

The values of gamma globulin and *Lactobacillus* counts were plotted as a linear graph. This shows evidence of a slight tendency for the gamma globulin levels to vary slightly inversely with the *Lactobacillus* counts. A plot of the two mean values for both gamma globulin and *Lactobacillus* counts shows such a slight tendency (Fig. 1).

DISCUSSION

The quantities of gamma globulin in human saliva are small. The maximum measured was about 10 mg. per cent; the smallest quantity was 2/10 mg. per cent. When compared with serum levels, the salivary levels of gamma globulin would represent hypogamma globulinemia (below 50 mg. per cent.)

The values of gamma globulin observed with *Lactobacillus* counts of 0–5,000 and 5,000+ show no correlation. Although the number of samples was small, it appears that a relatively constant level of gamma globulin, 2.55 mg. per cent, is found in the saliva of young men. The standard deviation from the mean, 0.4 mg. per cent, would give an estimate of range between 2.29 and 2.21 mg. per cent.

None of the subjects used in this experiment was caries-immune. The levels of gamma globulin are significant only in that it is present in saliva. The specific activity of such globulin for *Lactobacillus* must be studied. Green[2] has demonstrated that the globulin activity levels of saliva from caries-immune saliva are higher than in caries-susceptible saliva.

SUMMARY

Lactobacillus counts and gamma globulin levels in saliva from thirty-eight subjects were determined. The gamma globulin levels did not show any correlation with *Lactobacillus* counts. However, gamma globulin levels in saliva of non-caries-immune young men were constant and low.

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A *Geometrical Evaluation of Image Size in Dental Radiography*

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In radiographic projections the dimensions of an object are generally magnified and may even be distorted. Control of image size is therefore important both in dental practice and in dental research. The inherent tendency for magnification has been compensated in intraoral radiography by the use of Cieszynsky's "bisecting-angle" technique. In endodontics, special procedures have been suggested to determine the exact length of a tooth.[1]

Since faster films are available at present, an increasing use is made of a longer focus-film distance, as well as placement of the film parallel with the object.[2, 3] When this last condition cannot be fulfilled completely because of anatomical limitations, an attempt is made to achieve the closest possible parallelism of film and object.[4] The structural orientation in these projections is superior to that obtained with the "bisecting" technique.

A long focus-film distance and direction of the X-ray beam perpendicular to the film are basic principles in the standardization of views of the skull, developed by Broadbent[5] and Hofrath.[6] In later modifications of this method, the focus-film distance as well as the distance between the mid-sagittal plane and film are both kept constant when obtaining the lateral head radiograph. In this projection, the mid-sagittal plane is always parallel with the film casette.

Measurements on radiographs of this type are used extensively in growth studies and in investigations of the movements of the mandible. The effect of slight and unavoidable differences in head positioning has already concerned several investigators,[7–10] who attempted to determine the magnitude of the resulting errors by taking repeated radiographs of the same individuals, or skulls, and of specially constructed objects.

The purpose of the present study is to determine on a geometrical basis the amount of image magnification as a function of the angulation of film and object to the radiation; furthermore, to apply this function to currently used intraoral techniques in order to determine which method permits the greatest latitude of film placement and angulation to qualify as the most suitable one for clinical purposes without requiring positioning devices; and, finally, to investigate the influence of slight variations in the angulation of the mid-sagittal plane on linear and angular measurements in the lateral cephalometric radiograph.*

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* The term "cephalometric radiographs" is used for standardized radiographs of the skull not because it properly describes the method but because it has gained general acceptance.

EXPERIMENTAL METHODS AND RESULTS

Derivation of enlargement function.—A hypothetical situation for obtaining a radiographic image from an object is shown in Figure 1. For the purpose of discussion and derivation of the enlargement function, both object and film are drawn at different angles to the rays.

The rays emanate from the focal spot (F), which is assumed to be a point. The object (EK) is of length a and the image on the film (GI) of length a_1. The distance from the focal spot (F) to the object (EK) is expressed as FK and the distance from

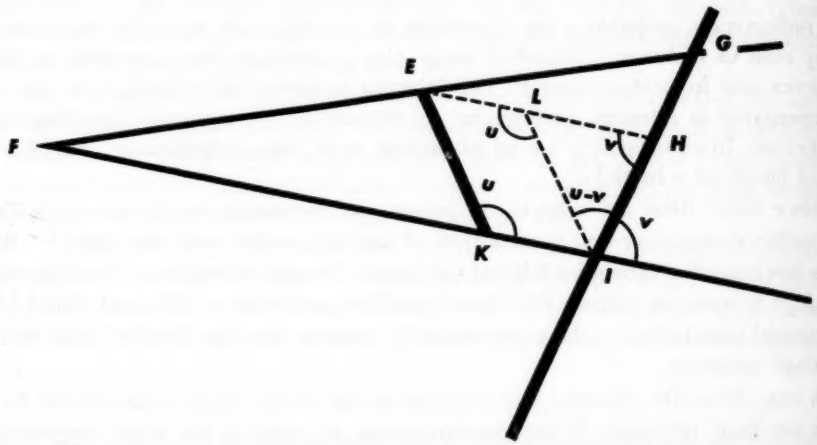

FIG. 1.—A hypothetical situation for obtaining a radiographic image of an object. F is the focal spot, EK the object, and GI the film plane. LI is drawn parallel with EK, and EH parallel with FI. The angles formed by a ray from F with the object and with the film are u and v, respectively.

the focal spot to the film as FI. The angles formed by the ray (FI) with the object and with the film plane are designated u and v, respectively. To calculate the image size (GI), the line EH is drawn parallel with FI and the line LI parallel with EK.

As shown in Figure 1, it follows that

$$LI = EK = a\,, \qquad EL = KI\,, \qquad \text{and} \qquad GI = a_1\,.$$

The lengths of HI and LH can be determined from $\triangle LIH$ because

$$\frac{LH}{\sin(u-v)} = \frac{IH}{\sin u} = \frac{LI}{\sin v}.$$

Therefore,

$$HI = LI \cdot \frac{\sin u}{\sin v} = a \cdot \frac{\sin u}{\sin v}, \tag{1}$$

and

$$LH = LI \cdot \frac{\sin(u-v)}{\sin v} = a \cdot \frac{\sin(u-v)}{\sin v}. \tag{1b}$$

GH is determined from $\triangle GEH$ and $\triangle GFI$ as follows:

$$\frac{GH}{GH+HI} = \frac{EL+LH}{FI}, \qquad \text{or} \qquad GH = \frac{HI \cdot (EL+LH)}{FI-EL-LH}.$$

The length of GH is found by substituting formulas (1) and (1*b*) for HI and LH, respectively:

$$GH = a \cdot \frac{\sin u}{\sin v} \cdot \frac{EL + a \cdot [\sin (u - v) / \sin v]}{FI - EL - a \cdot [\sin (u - v) / \sin v]}. \quad (2)$$

The length of the image (a_1) can be obtained from equations (1) and (2) as follows:

$$a_1 = GI = GH + HI = a \cdot \frac{\sin u}{\sin v} \cdot \frac{FI}{FI - EL - a \cdot [\sin (u - v) / \sin v]}. \quad (3)$$

This expression may be simplified if the focus-film distance (FI) is called D and the object-film distance (KI) is labeled d, namely,

$$a_1 = a \cdot \frac{\sin u}{\sin v} \cdot \frac{D}{D - d - a \cdot [\sin (u - v) / \sin v]}. \quad (4)$$

When object and film are parallel ($u = v$), the above equation is reduced to

$$a_1 = a \cdot \frac{D}{D - d}. \quad (4a)$$

Finally, if the rays are parallel, it will be seen that

$$a_1 = a \cdot \frac{\sin u}{\sin v}. \quad (4b)$$

Application of the function to intraoral techniques.—For the computation of image size, an object size of 24 mm. was chosen as an approximation of the length of a tooth. As usual in radiography, the angles and measurements will be expressed in relation to the central ray.

Image sizes obtained by variations in the angulations between the central ray and both the object and film planes are presented in Table 1, A. The determinations are based on the direction of the central ray through the apex of a tooth and perpendicular to the bisector between its long axis and the film plane (Fig. 2).

Data were obtained for object-film distances of 16 and 24 mm. with a focus-film distance of 20 cm. (8 inches). The object-film distance was 16 mm. when a "narrow" film (22×35 mm.) was used for radiography of the maxillary central incisor teeth, and the 24-mm. distance applied to the use of a "standard"-sized film (32×42 mm.) for this purpose. These object-film distances were selected on the basis of measurements from scale drawings of the adult palate.[4]

In the Table 1, B, data are given for picture magnification applicable to techniques calling for parallel film placement and a direction of the X-ray beam at right angles to the film. In such instances the central ray was aimed through the mid-point of the tooth, and the image length was the sum of the projections of the two halves of the tooth (Fig. 3). The findings in Table 1, B, pertain to a 40-cm. (16-inch) focus-film distance and object-film distances of 18 and 25 mm.

Thus findings of four different approaches for obtaining intraoral radiographs are presented in Table 1. For each technique the image size produced under optimal conditions of film placement and tube angulation is given, together with variations in the

image size resulting from changes of 10° from the optimal angulations between the central ray and the object and/or between the central ray and the film.

Means and standard deviations of the image sizes produced by different angulations for the four methods of projection were determined also. It is apparent from Table 1 that the variation in image size is greatest in the "bisecting" technique (S.D. = 1.74 and 2.79 mm.), while "parallel-film placement" techniques exhibit minimal variation (S.D. = 0.34 and 0.25 mm.). Consequently, the "bisecting" technique is less satisfactory in dental radiography.

TABLE 1

RADIOGRAPHIC IMAGE SIZES OF OBJECT 24 MM. IN LENGTH CALCULATED FOR VARIOUS ANGULATIONS AND VARIOUS FOCUS-FILM AND OBJECT-FILM DISTANCES

A. FOCUS-FILM DISTANCE 200 MM.

Angulation in Degrees between Central Ray and		16 Mm. Object-Film Distance (Image Size in Mm.)	Angulation in Degrees between Central Ray and		24-Mm. Object-Film Distance (Image Size in Mm.)
Object	Film		Object	Film	
60	100	21.13	50	110	19.74
60	110	21.75	50	120	21.03
60	120	23.08	50	130	23.20
70	100	23.34	60	110	22.63
70*	110*	23.98	60*	120*	24.00
70	120	25.38	60	130	26.72
80	100	24.96	70	110	24.94
80	110	25.56	70	120	24.40
80	120	27.05	70	130	29.00
Mean image size		24.03	Mean image size		24.18
Standard deviation		1.74	Standard deviation		2.79

B. FOCUS-FILM DISTANCE 400 MM.

Angulation in Degrees between Central Ray and		18-Mm. Object-Film Distance (Image Size in Mm.)	Angulation in Degrees between Central Ray and		25-Mm. Object-Film Distance (Image Size in Mm.)
Object	Film		Object	Film	
80	80	25.14	80	80	25.60
80	90	24.75	80	90	25.22
80	100	25.13	80	100	25.60
90	80	25.50	90	80	25.98
90*	90*	25.14	90*	90*	25.60
90	100	25.50	90	100	25.98
100	80	25.13	100	80	25.60
100	90	24.75	100	90	25.22
100	100	25.14	100	100	25.60
Mean image size		25.13	Mean image size		25.60
Standard deviation		0.34	Standard deviation		0.25

* These angulations are optimal for the technique.

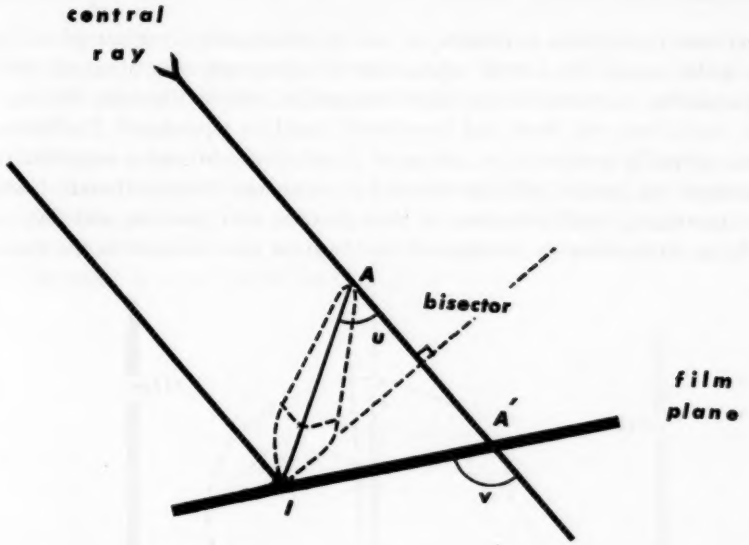

FIG. 2.—A radiographic image obtained with the "bisecting" technique. The central ray passes through the apex of a tooth at right angles to the bisector plane, forming angles u and v with the tooth and the film plane, respectively.

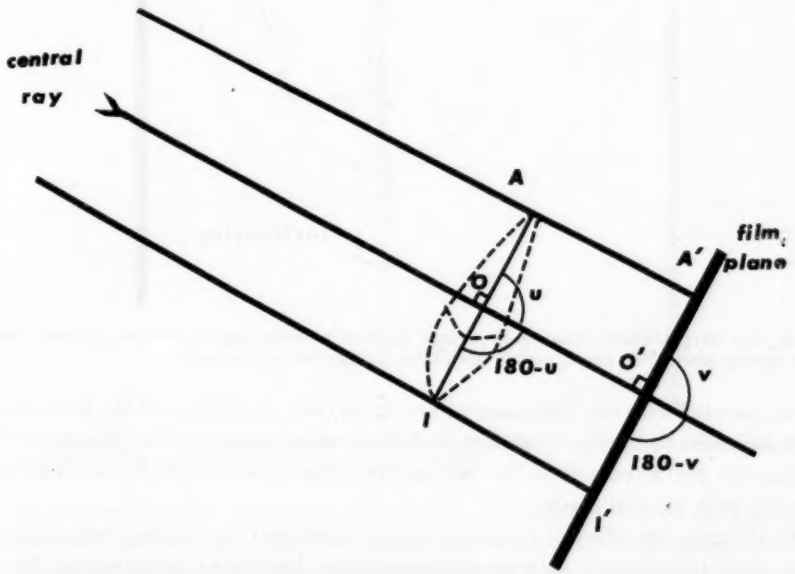

FIG. 3.—A radiographic image obtained with the "long-cone" technique. The central ray passes through the mid-point of a tooth, forming angle u with the tooth and angle v with the film. To determine the length of $A'O'$ consider angles u and v. To determine $I'O'$ consider angles $(180 - u)$ and $(180 - v)$.

Linear and angular measurements on lateral cephalometric radiographs.—The conditions under which the lateral cephalometric radiograph was obtained differ from those pertaining to intraoral techniques because the relation between the film casette and the central ray was fixed and therefore it could be reproduced. Furthermore, the head was carefully positioned by means of a cephalostat in such a way that the mid-sagittal plane was parallel with the film and at a constant distance from it. Under these circumstances, only small variations in head position were possible, and they occurred generally as inclinations or rotations of the head on axes situated in the mid-sagittal

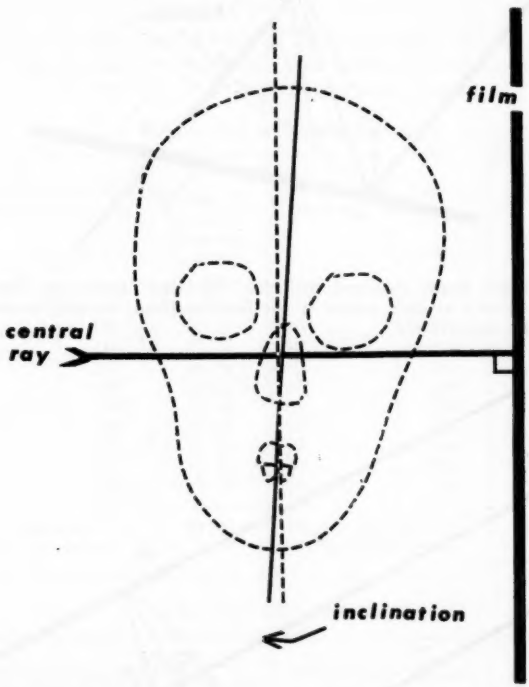

FIG. 4*a*.—Hypothetical situation of change in head position due to rotation around axis in the mid-sagittal plane. The axis is mainly horizontal (inclination of the head).

plane, parallel with the film plane (Figs. 4*a* and 4*b*). Rotations of the head on an axis at right angles to the film (different horizontal orientations) are not important for projections of characteristics in the mid-sagittal plane, provided that this plane remains parallel with the film plane.

To illustrate the effect of variations in head position (Figs. 4*a* and 4*b*) on the length of a projected distance between two anatomical landmarks as shown on the lateral cephalometric radiograph, an object size of 7 cm. was selected because this figure compared with the length of the frequently measured distance from nasion to sella turcica. For purposes of calculation it was assumed that the axis of rotation passed through the sella turcica while the focus-film distance was 159 cm. and the object-film distance was 9 cm. The fact that only the central ray was perpendicular to the film was disregarded

because the variation in this angulation was so small that it did not affect the results materially. It can be computed readily that under these conditions, at a 12-cm. distance from the central ray, the angulation of the rays deviates only 4° from the perpendicular.

The findings, summarized in Table 2, show that the changes in the length of the projection studied are very moderate (0.8 per cent or less) when the deviation from the optimal head position is no more than 5°. It should be noted, however, that the magnification in this projection is 6 per cent for objects anywhere in the mid-sagittal plane if this plane is parallel with the film.

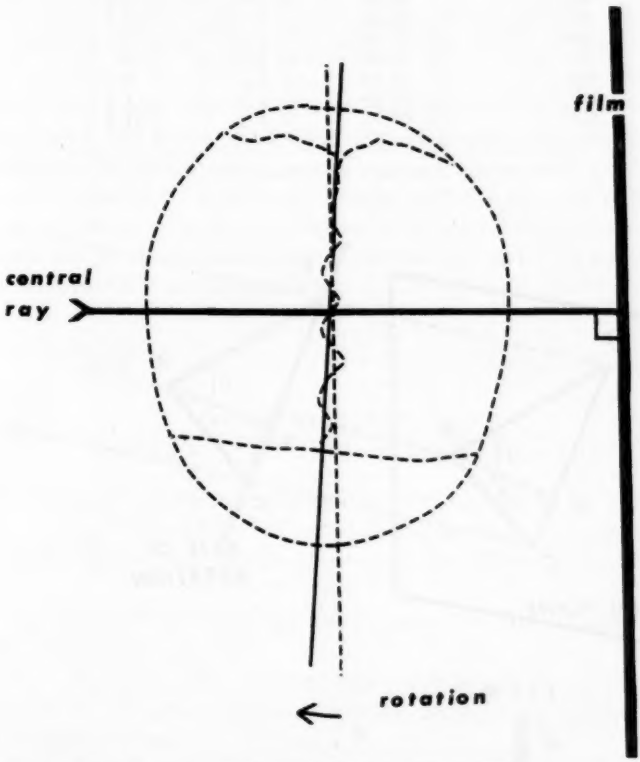

FIG. 4*b*.—Hypothetical situation of change in head position due to rotation around axis in the mid-sagittal plane. The axis is mainly vertical (rotation of the head).

To analyze the effect of rotation of the head on the magnitude of projected angles, it was assumed that both the angle to be studied and the axis of rotation were localized in the mid-sagittal plane. This situation is illustrated in Figure 5, where A is the angle which is to be projected. The legs of the angle are continued to intersect the axis of rotation at B and C; AD is the perpendicular from A on BC, and this line divides $\angle BAC$ in $\angle BAD$ and $\angle DAC$, of sizes y and x, respectively. The distances AB, AC, and AD are considered known. The same construction applies when one leg of the angle does not intersect the axis of rotation, because in such instances $(180 - A)$ may be

TABLE 2

RADIOGRAPHIC IMAGE SIZES OF OBJECT 70 MM. IN LENGTH CALCULATED FOR VARIOUS ANGULATIONS AND WITH FOCUS-FILM DISTANCE OF 1,590 MM. AND OBJECT-FILM DISTANCE OF 90 MM.

Angulation in Degrees of Mid-Sagittal Plane to Central Ray	Image Size (Mm.)	Enlargement (Per Cent)	Change of Image Size from 90° Projection (Per Cent)
60	62.79	−10.3	−15.4
70	68.65	− 1.9	− 7.5
80	72.50	+ 3.6	− 2.3
85	73.61	+ 5.2	− 0.8
90*	74.20	+ 6.0	.....
95	74.21	+ 6.0	0
100	73.68	+ 5.3	− 0.7
110	70.89	+ 1.3	− 4.4
120	65.75	− 6.1	−11.4

* This angulation is optimal for this technique.

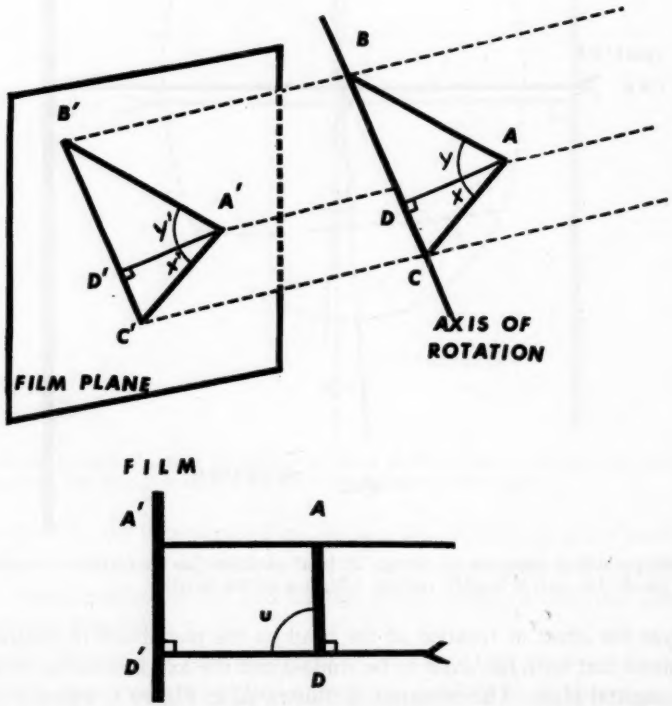

FIG. 5.—A hypothetical construction to determine the projection of angle *A*. Angles *B* and *C* are the intersections of the legs of this angle with the axis of rotation. *AD* is the perpendicular from *A* to *BC*, dividing $\angle BAC$ into $\angle BAD$ and $\angle DAC$, and $\triangle A'B'C'$ is the projection of $\triangle ABC$ on the film plane. The object is parallel with the film, as indicated in the cross-section at the bottom of the figure (*AD* is parallel with *A'D'*).

considered; moreover, if the angle has one leg parallel with the axis of rotation, the construction is obviously simpler.

The lengths of BD and CD are determined from $\triangle BAD$ and $\triangle DAC$, respectively. If $\triangle A'B'C'$ is the projection of $\triangle ABC$ and y' and x' denote the projections of y and x, then it can be shown—as long as the object and film planes are parallel and the focus-film and the object-film distances are the same as above—with application of equation (4*a*) that

$$\tan y' = \frac{B'D'}{A'D'} = \frac{BD}{AD} \cdot \frac{[159/(159-9)]}{[159/(159-9)]} = \frac{BD}{AD} = \tan y$$

and

$$\tan x' = \frac{D'C'}{A'D'} = \frac{DC}{AD} \cdot \frac{[159/(159-9)]}{[159/(159-9)]} = \frac{DC}{AD} = \tan x.$$

It follows that y and x and, therefore, $\angle A$ are unchanged in the projected image, and it may be concluded that angles localized in a plane parallel with the film are reproduced unchanged in the radiograph.

However, when plane BAC is no longer parallel with the film and perpendicular to the central ray but makes an angle of u degrees to the central ray instead of 90°, rotating around axis BC, as shown in Figure 6, functions (4) and (4*a*) can be applied to calculate the tangents of y' and x' as follows:

$$\tan y' = \frac{BD \cdot [159/(159-9)]}{AD \cdot \sin u \cdot \{159/[159-9-AD \cdot \sin(u-90)]\}}$$

and

$$\tan x' = \frac{DC \cdot [159/(159-9)]}{AD \cdot \sin u \cdot \{159/[159-9-AD \cdot \sin(u-90)]\}}.$$

Inspection of Figure 6 shows that projections of the length of line AD decrease in size when the angle u deviates from 90°, and this implies that the tangents of y' and x' increase in value. On the basis of this observation the following conclusions may be drawn:

1. Projections of angles of which the legs intersect the axis of rotation increase in size when the object plane is rotated.

2. Projections of angles where one leg does not intersect the axis of rotation decrease in size when the object plane is rotated, because the supplementary angle $(180 - A)$ increases in size.

3. Projections of right angles with one leg parallel with the axis of rotation are not changed by rotation.

In Figure 7 the problem discussed above is applied to an angle positioned in the mid-sagittal plane, where BC is the axis of rotation and A is the angle to be studied. Actual sizes for AB, AC, and AD are given in Table 3, in which the findings for the projections of the angle are presented as they change with deviations in the angulation of the object plane to the X-ray beam from 90°.

Five degrees deviation in angulation between the mid-sagittal plane and the central ray from the perpendicular does not affect the size of the projected angle appreciably, as seen in Table 3. Thus 5° leeway is acceptable when positioning the head in the

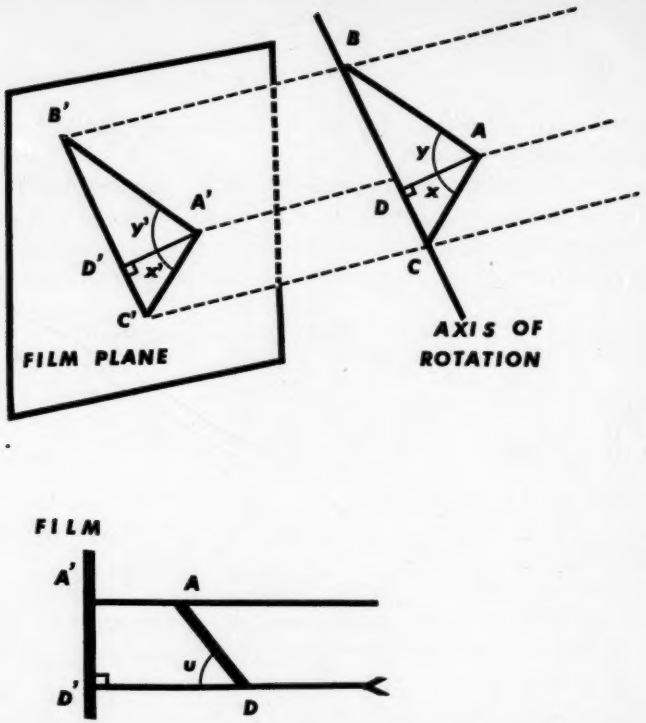

FIG. 6.—A hypothetical construction to determine the projection of angle *A*. Angles *B* and *C* are the intersections of the legs of this angle with the axis of rotation. *AD* is the perpendicular from *A* to *BC*, dividing $\angle BAC$ into $\angle BAD$ and $\angle DAC$, and $\triangle A'B'C'$ is the projection of $\triangle ABC$ on the film plane. The object plane is no longer parallel with the film, and it forms an angle with the X-ray beam, *u*, as indicated in the cross-section at the bottom of the figure.

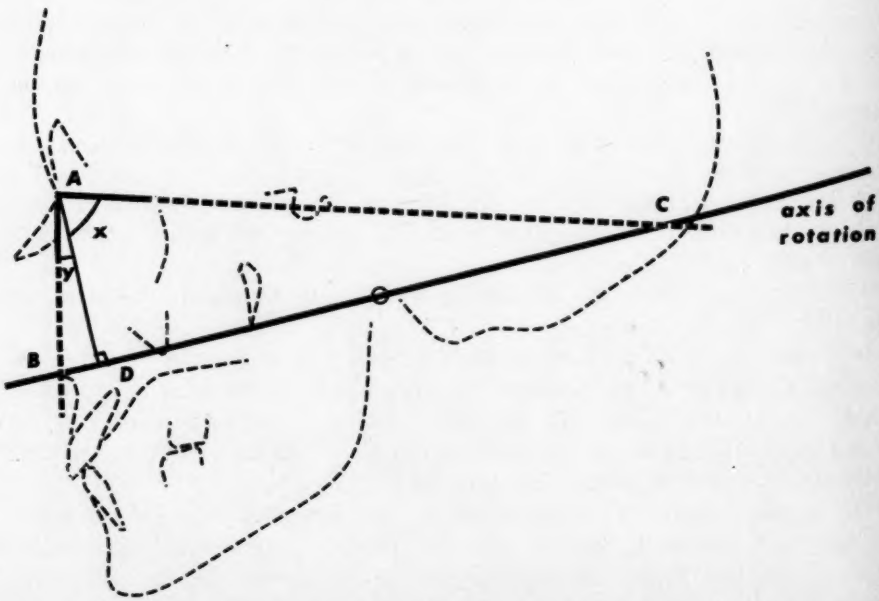

FIG. 7.—A construction to determine the projection of an angle in the mid-sagittal plane. *A* is the angle to be projected; *B* and *C* are the intersections of the legs of this angle with the axis of rotation; *AD* is the perpendicular from *A* to *BC*, dividing $\angle BAC$ into $\angle BAD$ and $\angle DAC$.

cephalostat, inasmuch as this deviation will not change the magnitude of the projected angles.

The findings in Table 3 are not affected materially when the central ray does not pass through D, if the deviation is within the limits actually encountered in lateral cephalography.

DISCUSSION

Function (4) is in accordance with the well-known dependence of the image size on the focus-film and object-film distances. Furthermore, it shows that changes in the angulation of the object to the X-ray beam from 90° reduce the size of the projected image, whereas image size is increased when the film plane is no longer parallel to the central ray.

TABLE 3*

CALCULATED MEASUREMENT OF THE RADIOGRAPHIC PROJECTION OF AN ANGLE (87°53′) OBTAINED UNDER VARIOUS CONDITIONS OF ANGULATION BETWEEN OBJECT AND FILM PLANE

Angulation in Degrees between Central Ray and Plane of Angle	y'	x'	$\angle B'A'C' = x'+y'$
80	16°07′	72°27′	88°34′
85	15°54′	72°12′	88°06′
90†	15°48′	72°05′	87°53′
95	15°49′	72°06′	87°55′
100	15°57′	72°15′	88°12′

* —$B'A'C'$ is the projection of $\angle BAC$; $AB = 53$ mm., $AC = 166$ mm., and $AD = 51$ mm. The focus-film distance is 1,590 mm., and the object-film distance is 90 mm.

† This angulation results in exact reproduction of the angle to be projected.

Interesting is the observation that changes in the image size are smallest for variations in the angulations between the X-ray beam and the film and/or the object when these angles assume values near 90°. This is a very desirable feature because it allows some latitude in film positioning and in angulation of the X-ray beam if parallel-film placement and projection at right angles to the film are maintained. Such latitude is necessary to obtain satisfactory results by a "freehand" technique in the dental office. The enlargement that has to be accepted is kept at a minimum by keeping the object-film distance short and the focus-film distance long.

The "bisecting-angle" technique shows greater change in image size for 10° variation from the optimum angulation than does the "parallel right-angle" method, as shown in Table 1. Moreover, the data demonstrate that there is little latitude in angulation of the X-ray beam when using the "bisecting-angle" technique. Therefore, it becomes of questionable merit to accept the poor structure orientation inherent in this technique when the only benefit—an isometric picture—is strongly dependent upon the correct determination of an imaginary bisecting plane. It seems preferable to use a longer focus-film distance and direction of the central ray perpendicular to the film and

parallel-film placement whenever this is consistent with reasonable object-film distances.

The image sizes presented in Table 2 relate to the validity of linear measurements on lateral cephalometric radiographs. An estimate of the variations in the lengths of projected distances between two landmarks may be obtained by taking a series of radiographs on the same subject and measuring the distance on each radiograph.

As part of the present study it was determined geometrically which changes would occur in the projected length of a distance when the mid-sagittal plane was not perpendicular to the central ray. The error in angulation of the mid-sagittal plane to the central ray is not likely to exceed 5°. The differences in the findings resulting from such a variation in head position are less than 1 per cent of the measurement obtained under optimal conditions.

It should be noted that a uniform magnification is expected throughout the picture on a geometrical basis if film and object planes are parallel. This finding is contrary to the observation of Adams[7] but is in accordance with the conclusions of Beek and van der Linden,[8] Hamernik,[11] and Kean.[12]

The functions developed in the present study have been used to determine the changes in the magnitude of the projection of an angle when varying the angulation between the central ray and the plane containing the angle and the axis of rotation. The results presented in Table 3 suggest that small differences ($\pm$ 5°) in the angulation of objects to the central ray do not produce measurable changes in the size of the projected angle. This observation is in accordance with the findings of Schwarz[9] and Nawrath.[10]

The conclusion that cephalometric radiography is a reliable tool for the study of both linear and angular changes as they occur during growth is supported by the present study, since these changes are not influenced by the generally encountered deviations in the alignment of the head in the cephalostat.

SUMMARY AND CONCLUSIONS

The relation between image size and the angulation of object and film to the direction of the X-ray beam is expressed in an equation. On the basis of this equation some currently used intraoral radiographic techniques are evaluated. Methods calling for film placement parallel to the teeth, with the central ray preferably directed perpendicular to the film plane, are found superior to the "bisecting-angle" technique for producing uniform pictures of a tooth.

The errors introduced into linear and angular measurements on lateral cephalometric radiographs by unavoidable variations in the relation of the mid-sagittal plane to the central ray are negligible if these variations are 5° or less. Thus cephalometric radiography is a suitable means for the study of dimensional changes in the skull.

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Periodontal Disease in India: Report of an Epidemiological Study

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The high prevalence of periodontal disease in India has been referred to in a number of publications.[1-10] These reports, supported by observations of World Health Organization (WHO) consultants, stimulated WHO, in co-operation with the government of India, to undertake an epidemiological investigation of the India periodontal disease problem in mid–1957. This co-operative investigation of the nature and extent of periodontal disease in India was preceded by a seminar on modern concepts of the disease and investigative methods. During the seminar, which was held in Bombay from July 22 to August 3, 1957, time was devoted to standardizing methods for examining and recording clinical evidence of periodontal disease and to developing final details for the study.

Following the seminar, a periodontal disease field study was conducted in Bombay and in a nearby rural area. Two different procedures were used on each examinee to obtain periodontal disease data. Data obtained by using Russell's[11] procedure form the basis of this report. Data based on the other procedure (Ramfjord's method[12]) will be presented by Ramfjord in a separate publication.

EXPERIMENTAL METHODS

The study group comprised 1,750 males, eleven to thirty years of age, 1,678 of whom were eleven, thirteen, fifteen, or seventeen years of age. The examinations completed during a 2-day standardization period were deleted, leaving 1,613 males, aged eleven, thirteen, fifteen, and seventeen, and 63 males, eighteen to thirty years of age, for analysis. Eight hundred and two of this group came from urban public schools in Bombay and the remainder from a rural area known as Basein, approximately 50 miles from Bombay. (All persons over seventeen years of age were from this rural area.)

Young people were selected for study, in order to ascertain when periodontal disease has its inception and to observe early stages of alveolar bone loss. Alternate ages were selected to provide a greater age span with a minimum number of examinations. The study was limited to males, in order to avoid including "between-sex" variations. The urban group was of low socio-economic status but did not represent the poorest segment of the population, since few from this latter group are found in school. The rural group was essentially of comparable socio-economic status. The economy of the Basein area is based primarily on agriculture, bananas, coconuts, and rice being the major products. The area is connected with the city of Bombay by a railroad but otherwise is rather isolated.

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The examination team participating in the portion of the study upon which this report is based included nine dentists—eight from India and one from the United States. The following data were recorded: (1) identifying information, (2) religion, (3) language, (4) method of cleansing the teeth (brush, finger, or stick), (5) cleansing material used, (6) frequency of cleansing the teeth, and (7) scores for oral debris, oral calculus, and periodontal disease.

Conditions of the periodontal structures were recorded according to Russell's method.[11] This method of scoring periodontal disease is based on positive recordings for obvious mild gingivitis, as well as for overt signs of far-advanced destruction of the structures supporting the teeth. Therefore, the terms "periodontal disease" and "periodontal scores" as used in this report refer to clinical evidence of these or intermediate conditions. The method used for scoring oral debris and oral calculus and for classifying oral hygiene status was the method described by Greene and Vermillion.[13] According to this method, the lower the score, the better the oral hygiene status. The non-clinical information was obtained by interviewers who also acted as interpreters and recorders.

Examinations were done with the aid of portable dental chairs, mouth mirrors, and explorers. Electricity was not available in several of the schools. Therefore, in order to maintain uniform lighting, all examinations were done without artificial lighting. The examinations completed on the first 2 days were closely supervised and observed by the author. As previously mentioned, the examinations completed during these 2 days were used for standardization purposes; hence these data are not included in the report.

Since several examiners with different backgrounds were to participate in data collection in the India study, an effort was made to rotate examiners so that each would examine comparable numbers in each age group in the urban and rural segments of the study. As a result of this effort, a fairly equitable distribution of examiners' observations was realized.

Close inspection of the data collected by individual examiners revealed little "inter-examiner" variation, with the exception of the debris recordings. This variation in the debris scores may have been due, in part, to incomplete standardization and to normal patient variation in the few people examined by each examiner in each age in each of the urban and rural groups. The periodontal disease and oral hygiene scores collected by each examiner showed the same relationships between the urban and rural populations. Since each examiner's observations were well distributed among the groups, there appeared to be no valid reason for excluding data collected by one or more examiners; therefore, the data collected by all examiners were combined for analysis.

For comparative purposes, 577 white males, aged eleven, thirteen, fifteen, and seventeen years, were examined by the author in the public schools in Atlanta, Georgia. The majority of these children were from very low socio-economic areas of the city. The same basic information was recorded, and the some methods and equipment were used in Atlanta as in India.

RESULTS

Periodontal disease and oral hygiene scores by age for the urban and rural groups examined in India are listed in Table 1. The periodontal scores remained relatively stable for ages eleven to seventeen years. However, the scores were considerably higher

for ages nineteen and above. The debris index fluctuated slightly but was essentially the same for all ages through thirty. The calculus index consistently increased with age in both the urban and the rural group. The oral hygiene index increased with age but not so rapidly as the calculus index.

The periodontal disease scores recorded for the low socio-economic group in urban Bombay were consistently lower than for the same ages studied in the nearby rural area of Basein (Fig. 1). These differences were statistically significant* for each of the

TABLE 1

PERIODONTAL DISEASE, ORAL DEBRIS, CALCULUS, AND ORAL HYGIENE SCORES BY AGE FOR PERSONS EXAMINED IN URBAN INDIA (BOMBAY) AND RURAL INDIA (BASEIN)

PLACE AND AGE	NUMBER EXAMINED	MEAN INDEX SCORES			
		Periodontal Disease	Debris	Calculus	Oral Hygiene
Urban:					
11.....	304	0.76±0.02*	2.84±0.05*	1.57±0.06*	4.41±0.02*
13.....	259	0.68±0.03	2.78±0.05	1.99±0.07	4.77±0.03
15.....	194	0.77±0.04	2.63±0.08	2.16±0.09	4.79±0.11
17.....	45	0.75±0.07	2.63±0.14	2.40±0.20	5.03±0.08
11–17.....	802	0.73±0.02	2.76±0.04	1.89±0.04	4.65±0.05
Rural:					
11.....	208	0.98±0.03	3.20±0.08	2.08±0.08	5.28±0.11
13.....	210	0.95±0.03	3.06±0.07	2.43±0.09	5.49±0.12
15.....	206	0.96±0.03	2.87±0.06	2.66±0.09	5.53±0.11
17.....	187	1.05±0.04	2.88±0.07	2.82±0.09	5.70±0.12
11–17.....	811	0.98±0.02	3.00±0.04	2.49±0.04	5.49±0.06
18.....	7	1.00±0.12	2.67±0.46	2.73±0.36	5.40±0.50
19–22.....	18	1.36±0.17	2.84±0.21	3.33±0.18	6.17±0.30
23–26.....	27	1.45±0.12	2.85±0.16	3.37±0.22	6.22±0.29
27–30.....	11	2.00±0.51	2.50±0.39	4.09±0.33	6.59±0.65

* Standard error.

ages eleven, thirteen, fifteen, and seventeen. Corresponding to these high periodontal scores in the rural area were higher debris and calculus scores for each of these four ages. The differences between the urban and rural debris and calculus scores were statistically significant for each of the ages eleven, thirteen, and fifteen, and the rural oral hygiene index was significantly higher than the urban for each of the four ages.

A summary of the findings from the comparative Atlanta, Georgia, study is shown in Table 2. As in the India study, the periodontal disease scores were essentially the same for each age but were significantly lower than those for comparable age groups in India (Fig. 2). The calculus scores in the Atlanta group were significantly lower than the calculus scores in the India counterparts (Fig. 3). However, the debris scores in the Atlanta group were consistently higher than the ones recorded in India. This difference in debris scores was significant for the ages eleven, thirteen, and fifteen. Like

* For this report, a difference is considered significant if it could not be expected to occur by chance more often than one time in 100 ($P \geqq 0.01$).

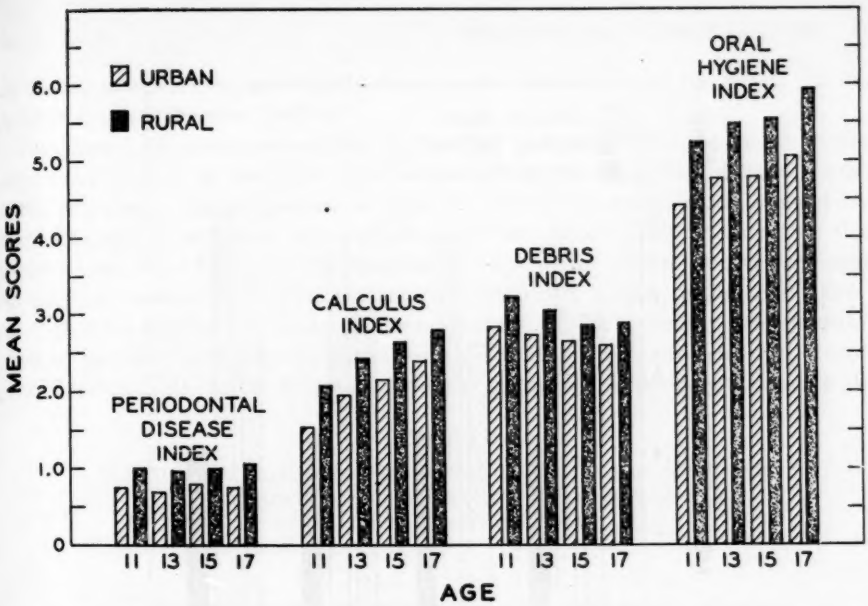

FIG. 1.—Periodontal disease, oral calculus, debris, and oral hygiene indexes by age for urban and rural India.

TABLE 2

PERIODONTAL DISEASE, DEBRIS, CALCULUS, AND ORAL HYGIENE INDEXES BY AGE FOR 577 WHITE MALES EXAMINED IN ATLANTA, GEORGIA

AGE	NUMBER EXAMINED	MEAN INDEX SCORES			
		Periodontal Disease	Debris	Calculus	Oral Hygiene
11.....	165	0.41±0.03*	3.28±0.07*	0.13±0.02*	3.41±0.08*
13.....	142	0.45±0.03	3.22±0.07	0.27±0.04	3.49±0.09
15.....	169	0.45±0.03	3.13±0.06	0.31±0.04	3.44±0.08
17.....	101	0.42±0.04	2.70±0.09	0.39±0.07	3.11±0.14
11-17.....	577	0.43±0.01	3.12±0.04	0.26±0.02	3.38±0.05

* Standard error.

TABLE 3

NUMBER AND PER CENT OF PERSONS HAVING OVERT GINGIVAL INFLAMMATION AND NUMBER AND PER CENT HAVING OBVIOUS PERIODONTAL POCKETS BY AGE IN URBAN AND RURAL INDIA AND ATLANTA, GEORGIA

AGE	NUMBER EXAMINED		PERSONS WITH POSITIVE PERIODONTAL DISEASE SCORES				PERSONS WITH OBVIOUS PERIODONTAL POCKETS			
	India	Atlanta	India		Atlanta		India		Atlanta	
			No.	Per Cent	No.	Per Cent	No.	Per Cent	No.	Per Cent
11.....	512	165	500	97.7	149	90.3	1	0.2	0	.....
13.....	469	142	443	94.5	135	95.1	2	0.4	1	0.7
15.....	400	169	390	97.5	161	95.3	4	1.0	0	.....
17.....	232	101	230	99.1	86	85.1	14	6.0	2	2.0

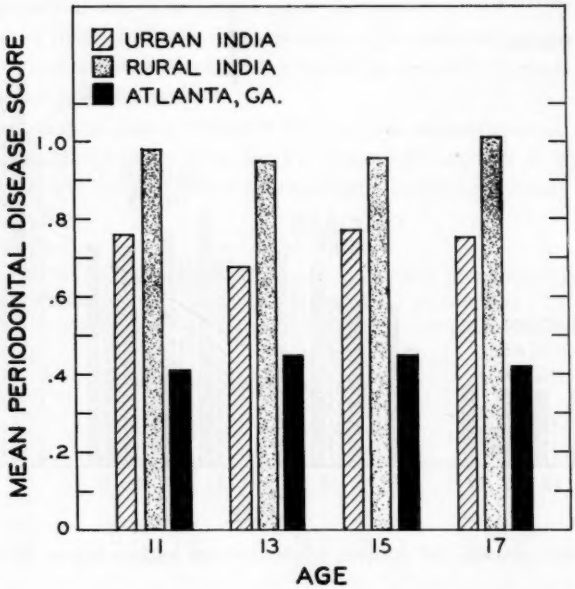

FIG. 2.—Periodontal disease scores by age for urban India, rural India, and Atlanta, Georgia

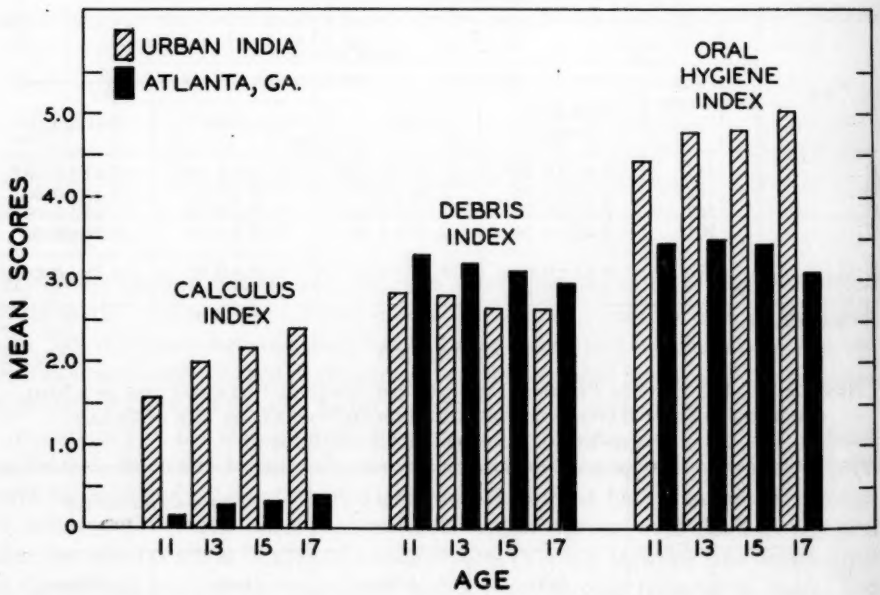

FIG. 3.—Oral calculus, debris, and oral hygiene indexes by age for urban India and Atlanta, Georgia

the Indian debris scores, the Atlanta debris scores were higher for the eleven-year-old children than for the other children.

The eleven- to seventeen-year-old Indians did not demonstrate as many obvious periodontal pockets as had been anticipated from reviewing previous articles relating to the periodontal disease problem in India.[3–5] Most of the periodontal disease scores below the age of seventeen were reflections of soft-tissue inflammation rather than alveolar bone loss (Table 3). Ninety-seven per cent of the eleven- to seventeen-year-old persons examined had overt evidence of periodontal disease, while fewer than 2 per cent of the total had obvious periodontal pockets. All the 63 persons over seventeen years of age had overt gingival inflammation, and 19, or 30.2 per cent, had obvious periodontal pockets. In the Atlanta sample the prevalence of periodontal disease also

TABLE 4

ORAL HYGIENE INDEX RELATED TO PERIODONTAL DISEASE SCORES FOR ALL MALES AGED ELEVEN TO SEVENTEEN YEARS, EXAMINED IN INDIA AND ATLANTA, GEORGIA

Oral Hygiene Index	India		Atlanta	
	No. of Persons	Mean Periodontal Disease Score	No. of Persons	Mean Periodontal Disease Score
0.0– 1.0.....	5	0.08*	4	0.08*
1.1– 2.0.....	38	0.25±0.05†	49	0.10 ±0.08†
2.1– 3.0.....	113	0.43±0.03†	210	0.26‡±0.01†
3.1– 4.0.....	276	0.60±0.02†	188	0.46‡±0.02†
4.1– 5.0.....	400	0.75±0.02†	81	0.65‡±0.03†
5.1– 6.0.....	388	0.94±0.02†	30	1.05 ±0.06†
6.1– 7.0.....	227	1.13±0.03†	8	1.23*
7.1– 8.0.....	96	1.30±0.04†	5	1.56*
8.1– 9.0.....	52	1.52±0.06†	1	1.90*
9.1–10.0.....	17	1.76*	1	1.80*
10.1–11.0.....	1	2.00*	0	

* Standard errors not calculated for fewer than 30 cases.
† Standard error.
‡ Significantly lower than India.

was very high (92 per cent); however, only 3 persons out of 577 exhibited obvious periodontal pocket formation.

All examinations of the eleven- to seventeen-year-olds in the India study were grouped according to their oral hygiene index scores, and the mean periodontal disease scores for each group are shown in Table 4 and Figure 4. The Atlanta data are treated in the same manner. For groups with similar oral hygiene indexes, the differences between the Atlanta and India periodontal disease scores were statistically significant for the groups with oral hygiene indexes between 2.1 and 5.0. The number of cases in the other oral hygiene groups was too small for reliable tests of significance. In both India and Atlanta there was a striking direct relationship between the oral hygiene and periodontal disease scores.

All India examinations in the eleven- to seventeen-year age group were combined according to their periodontal disease scores, and the mean debris, calculus, and oral hygiene scores for each of the periodontal disease groups are shown in Table 5 and

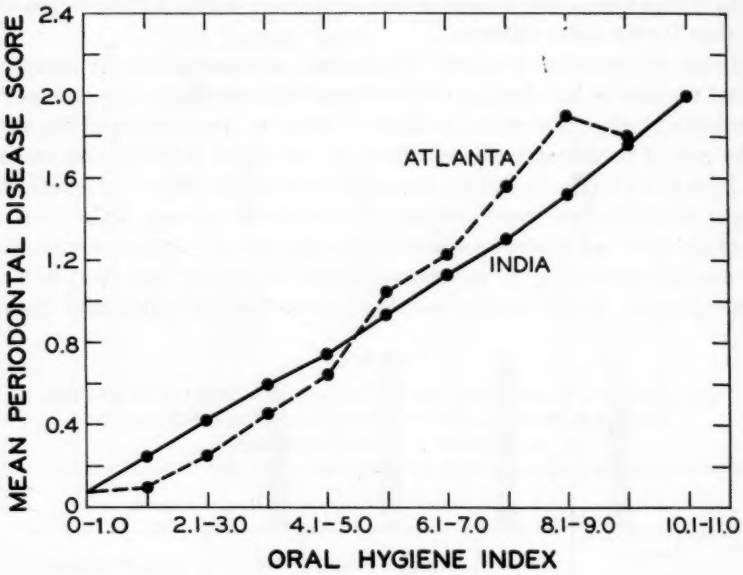

FIG. 4.—The oral hygiene index compared with periodontal disease scores for eleven- to seventeen-year-old males in urban and rural India and Atlanta, Georgia.

TABLE 5

MEAN PERIODONTAL DISEASE AND ORAL HYGIENE SCORES FOR PERSONS AGED ELEVEN TO SEVENTEEN YEARS EXAMINED IN URBAN AND RURAL INDIA

PERIODONTAL DISEASE SCORES	NUMBER EXAMINED	MEAN INDEXES		
		Debris	Calculus	Oral Hygiene
0.0–0.1	85	2.09	.84	2.93
0.2–0.3	153	2.48	1.29	3.77
0.4–0.5	226	2.76	1.54	4.30
0.6–0.7	258	2.90	1.91	4.81
0.8–0.9	234	2.82	2.26	5.08
1.0–1.1	235	2.98	2.47	5.45
1.2–1.3	154	3.08	2.83	5.92
1.4–1.5	146	3.16	3.04	6.20
1.6–1.7	58	3.20	3.35	6.55
1.8–1.9	46	3.65	3.48	7.13
2.0–2.1	10	3.79	4.36	8.15
2.2 and over*	8	†	†	†

* Scores range from 2.2 to 3.7.

† Means not calculated because of range of periodontal disease scores.

Figure 5. The calculus and debris scores and the oral hygiene index increased with each increase in periodontal disease score; however, the calculus scores increased at a more rapid rate than did the debris scores. There was a sharp rise in the oral hygiene index with each increase in the periodontal disease score.

The interrelationships between age, debris, calculus, oral hygiene, and periodontal disease indexes were calculated by the simple linear, partial, and multiple-correlation techniques. The following factors in both the India and the Atlanta group were significanty correlated:* scores for debris and periodontal disease, calculus and periodontal disease, and the oral hygiene index and periodontal disease.

The five characteristics recorded in India—religion, language, method of cleansing

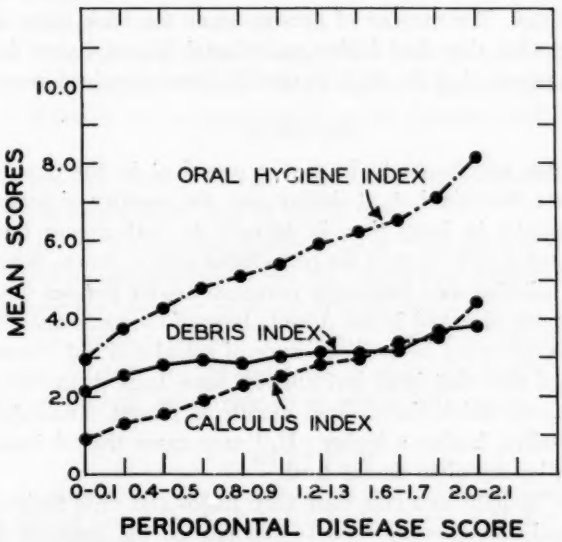

FIG. 5.—Mean oral hygiene scores compared with periodontal disease scores for persons aged eleven to seventeen years examined in urban and rural India.

the teeth (brush, finger, or stick), cleansing material used, and frequency of cleansing the teeth—were compared with the clinical scores to determine possible associations. Analysis of these five characteristics individually did not show a significant association with the oral hygiene or periodontal disease scores.

In the group studied, the most commonly used method of cleansing the teeth was rubbing the index finger over the teeth and gums. Of those examined, 635, or 78 per cent, of the rural persons and 755, or 94 per cent, of the urban persons used this method. Only 6 per cent of the total sample used a tootbrush for maintaining oral hygiene. Both urban and rural toothbrush users had somewhat lower debris and calculus scores and tended to have lower periodontal disease scores than those who used the finger as a means of cleansing the teeth. These calculations were based on very small numbers of persons and should be considered only as indicating trends worthy of further study. The differences were not statistically significant. The scores for periodon-

*For this report, correlations were considered significant when the *P*-value was less than 0.001.

tal disease, debris, and calculus for persons who used the stick fluctuated considerably and indicated no clear-cut relationship.

Some of the cleansing materials used by those examined in this study were charred tobacco, charcoal, coal powder, cow dung, rice husks, salt, snuff, soil, and various commercial or proprietary dentifrices in the form of pastes and powders. The number of persons using each of these materials was so small that they were categorized according to whether they used proprietary or non-proprietary dentifrices. With this grouping, the differences in clinical scores between the groups were not significant.

According to the data collected, less than 20 per cent of the study group cleansed their mouths more frequently than once per day. There was little difference between the periodontal disease scores of those who cleansed once per day and those who cleansed twice per day. The number of persons using the stick twice daily was very small, but the fact that they had higher periodontal disease scores for each of the four ages studied suggests that the stick, as used by those examined, may be traumatic.

DISCUSSION

Periodontal disease was found to be highly prevalent in the populations studied in India and Atlanta, Georgia, but, at similar ages, the severity of periodontal disease was significantly greater in India than in Atlanta. In both groups the oral hygiene scores were associated positively with the periodontal disease scores. Remarkably large deposits of dental calculus were unusually prevalent among persons studied in India. Very little calculus was observed in the Atlanta boys of the same age. An explanation for this tremendous difference in the abundance of calculus is not apparent, although it has been observed that that betel leaf chewers have large quantities of calculus.[14] Chewing betel leaf, on which slaked lime usually is placed, stimulates the flow of saliva. Stimulated saliva, having a higher pH,[15] may cause the calcium salts to drop out of solution and be deposited on the teeth.

Bery stated that "Indians as a rule wash their hands and rinse their mouths before and after every meal, not allowing food to stagnate on the teeth."[16] The fact that the Indians rinse their mouths frequently and fewer than half of the Atlanta group brushed their teeth one or more times a day may account for the lower debris scores in India.

The oral hygiene and periodontal disease scores are correlated in a positive direction in the India and Atlanta study groups. When the India and Atlanta groups of similar oral hygiene status are compared, the India periodontal disease scores are significantly higher than the Atlanta scores. This is true for groups having oral hygiene indexes between 2.1 and 5.0. Other groups were too small for valid comparison. This difference suggests that either the inflammatory response to debris and calculus in the Indian people is greater than in Atlanta or that there are other important irritants or altering factors active in India which were not active in the Atlanta study group.

In the presence of the unusually abundant calculus deposits and the prevalence of severe gingival inflammation, there was little overt clinical evidence of alveolar bone loss in the eleven- to seventeen-year-olds in India.

It had been hypothesized while designing the study that the health habits of individuals in the various language and religious groups might differ sufficiently to cause

significant variations in the oral hygiene and periodontal disease scores. However, these data do not substantiate the original hypothesis. Nevertheless, the number of cases in the subgroups (urban, rural, age, and language or religion) were so small that the validity of this hypothesis cannot be ruled out completely on the basis of these data.

SUMMARY

1. An epidemiological study of periodontal disease in and around Bombay, India, and in Atlanta, Georgia, was conducted in 1957. The study involved 1,613 males, aged eleven, thirteen, fifteen, and seventeen, in India and 577 males of the same ages in Atlanta. In addition, 63 Indian males, eighteen to thirty years of age, were examined in the rural area near Bombay.

2. Periodontal disease was highly prevalent in both groups but was significantly more severe in India than in Atlanta. Oral calculus was more abundant in the India group, while oral debris was more plentiful in the Atlanta group than in the urban India sample.

3. The rural persons studied in India had more severe periodontal disease, more calculus, and more debris than those in urban India.

4. When groups of similar oral hygiene status were compared, there still was a statistically significant difference between the periodontal disease scores in India and Atlanta, suggesting the presence of other altering factors.

5. The language, religion, method of cleansing the teeth, materials used in cleansing the teeth, and frequency of cleansing had no apparent significant association with the oral hygiene or periodontal disease scores in the India study.

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*Selective Colorimetric Determination of Local Anesthetics Containing a Tertiary Amine**

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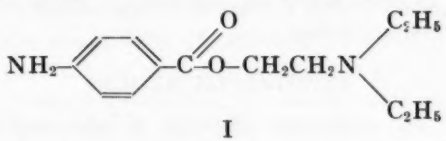
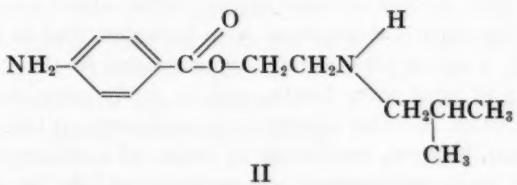
In an earlier publication from this laboratory[1] the need for new, selective methods for the determination of certain local anesthetic compounds was discussed. The limitations of the classical methods of analysis and of some of the newer instrumental procedures were noted, and a colorimetric method applicable to anesthetics having a primary amine was described. A more recent publication[2] described the extension of a procedure reported earlier by Sass, Kaufman, Cardenas, and Martin[3] to the analysis of small quantities and low concentrations of lidocaine. This procedure, which employs a reagent containing *cis*-aconitic anhydride, appears to present a new approach to the selective analysis of anesthetic compounds which contain a tertiary amine group without interference from primary or secondary amines.

Other colorimetric methods presently available, such as diazotization-coupling methods,[4-7] Schiff base reactions,[8, 9] nitrous acid procedures,[10-13] acylation reactions,[14, 15] and the sodium 1,2-naphthoquinone-4-sulfonate method,[1] are generally selective for compounds containing either a primary or a secondary amine. However, no good selective colorimetric method for the assay of tertiary amines has previously come to our attention.

The need for a selective method for the determination of tertiary amine compounds arose recently as a result of the marketing of a relatively new local anesthetic combination in dentistry. This dosage form‡ consists of a mixture of procaine hydrochloride (2′-diethylaminoethyl 4-aminobenzoate hydrochloride) and butethamine hydrochloride (2′-isobutylaminoethyl 4-aminobenzoate hydrochloride). The structural formulas for procaine (I) and for butethamine (II) are illustrated:



I



II

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* Analysis of Local Anesthetics. V. For the preceding paper in this series see *Analytical Chemistry*.[16]

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‡ Graham Chemical Corporation markets a solution for injection containing 2.0 per cent of procaine hydrochloride and 1.25 per cent butethamine hydrochloride under the brand name of Duocaine.

These two anesthetic agents have almost identical physical and chemical properties, which makes their individual determination in mixtures most difficult. The two compounds have very similar structural formulas and identical molecular weights and empirical formulas. Furthermore, the drugs have nearly identical pK values and almost superimposable ultraviolet absorption spectra. These properties preclude individual determinations by conventional acid-base titration methods or ultraviolet spectroscopy. The infrared spectra of the two compounds differed significantly in some regions, but the differences were deemed insufficient for use in a quantitative method.

Complete separation of the drugs via ion-exchange resins or paper electrophoresis was unsuccessful, probably because of the close similarity between the pK values. Chromatographic separation was sharp[16] but did not allow quantitative determinations.

From the standpoint of chemical stucture, as seen from the illustrations, procaine and butethamine differ in only one significant respect; that is, the procaine molecule contains a tertiary amino group in addition to a primary amine, while the butethamine molecule includes a secondary amino group in addition to a primary amine. For this reason, interest, of necessity, was focused on the secondary and tertiary amine functional groups.

An attempt to separate the secondary amine by a reaction described by Duke[17] was approximately 80 per cent complete and was not considered sufficient for quantitative purposes.

Because of the limitations of those chemical methods which are presently commonly employed for tertiary amines, the colorimetric reaction with *cis*-aconitic anhydride appeared particularly useful for this system. After appropriate modifications of the earlier procedure, the method discussed herein was found to give highly satisfactory results in the case of this particular mixture and, moreover, to be a generally useful method for the determination of a number of other tertiary amine anesthetics, particularly in those cases where only micro quantities or extremely dilute solutions are available. In no case was it found that anesthetics having primary or secondary amino groups, but no tertiary amine, give rise to interference in this procedure. This was found to apply even in cases where the non-tertiary amine compound is present in greater than tenfold concentrations.

EXPERIMENTAL METHODS

Chemicals, reagent, and apparatus.—Samples of local anesthetics were generously provided by the same sources as noted earlier.[1] Other chemicals, the preparation of the reagent, and the apparatus employed have been described in a previous publication.[2] In addition, a spectrophotometer* was employed in this case, both to check absorbance values at peak wave lengths and to verify complete extraction of the various anesthetic bases. All other absorption measurements at individual wave lengths in the visible region, however, were made by means of a colorimeter† with a No. 54 filter. An infrared spectrophotometer‡ was employed to take the infrared absorption curves.

Procedure.—(*a*) Preparation of Standard and Unknown Sample Solutions.—Accurately measured samples of the unknown aqueous solution and of an aqueous stock

* Beckman DU. † Klett-Summerson. ‡ Perkin-Elmer Model 21.

standard solution of procaine hydrochloride or other tertiary amine of interest were transferred to individual small separatory funnels. Each solution was made alkaline with 10 per cent ammonia solution and was promptly extracted with an accurately measured volume of toluene. The bulk of the toluene layer was removed, centrifuged briefly, and an aliquot was withdrawn for dilution to the desired concentrations. Measured volumes of the toluene extract of the standard solution were diluted to give final tertiary amine concentrations which covered the usable range of the method, as noted in Table 1; the extract from the unknown solution was diluted to give a final amine concentration which would fall within this range.

b) Color Development.—The characteristic color was developed and measured in the same manner as previously described. The reading obtained with the unknown solution was compared with a graph of the standard curve, which had been prepared by plotting the readings obtained with the standard solutions versus the respective amine concentrations.

TABLE 1

USABLE LIMITS OF ANESTHETIC CONCENTRATION

ANESTHETIC	TOTAL MICROMOLES PER SAMPLE	TOTAL MICROGRAMS PER SAMPLE	
		Base	Salt*
Butacaine	0.2 –1.2	61.3–367.7	71.1–426.6
Lidocaine	.1 –1.0	23.4–234.5	27.1–270.8
Metabutoxycaine	.06–1.0	18.5–308.4	20.7–344.9
Piperocaine	.2 –1.0	51.2–256.3	58.5–292.8
Procaine	.1 –1.0	23.6–236.3	27.3–272.8
Propoxycaine	.1 –1.0	29.4–294.4	33.1–330.8
Tetracaine	0.2 –1.6	52.9–423.0	60.2–481.3

* All as hydrochloride salts except butacaine as the sulfate.

c) Determination of Butethamine Hydrochloride.—The ultraviolet absorption peaks of both procaine hydrochloride and butethamine hydrochloride in aqueous solution occurred at 290 mμ. The absorptivity (1 per cent, 1 cm.) of procaine hydrochloride was about 660, while that of butethamine hydrochloride was about 631. If, therefore, the concentration of procaine hydrochloride, in a mixed solution of butethamine hydrochloride and procaine hydrochloride, had been determined using the *cis*-aconitic anhydride reagent, it would then have been possible to determine the concentration of butethamine hydrochloride. This was done by taking an absorbance reading at 290 mμ and then applying the following equation: $B = 1/631\ (A - 660P)$, where B is the butethamine hydrochloride concentration, P is the procaine hydrochloride concentration, and A is the observed absorbance reading.

RESULTS AND DISCUSSION

Extraction into toluene.—The accuracy of the method was dependent upon complete extraction of the respective tertiary amine anesthetics into a single portion of toluene after the aqueous solution had been made alkaline with ammonia. The completeness of extraction was checked by the following procedure. Initially the absorb-

ance of a diluted aliquot of an aqueous solution was determined; a portion of the aqueous solution was then made alkaline and extracted with toluene as in the assay procedure; an aliquot of the toluene extract was, in turn, extracted with diluted hydrochloric acid; the hydrochloric acid extract was buffered to a pH of about 6; the resulting solution was suitably diluted and its absorbance determined. The absorbance value of the original solution was then compared with the absorbance value obtained with the solution which had been subjected to the extraction, after making appropriate corrections for the dilutions involved. In the case of each tertiary amine anesthetic, it was found that at least 99 per cent of the material was carried through the dual extraction procedure, indicating that a single toluene extraction can be regarded as quantitative for the purposes of this assay.

Optimum heating period.—Each tertiary amine anesthetic was studied to establish the optimum length of heating. While minor differences were noted between the different compounds, 45 seconds was found to be satisfactory in all cases.

Wave length of measurement.—An absorption curve in the visible region, which was obtained with the procaine color complex, is shown in Figure 1. It can be seen that

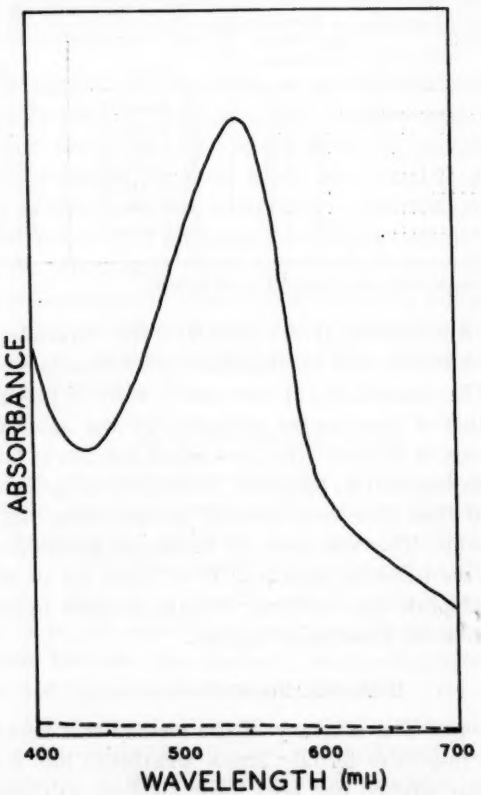

FIG. 1.—Visible absorbance curve of *cis*-aconitic anhydride color complex; —— procaine complex; - - - - - reagent blank.

the wave length of maximum absorption occurs at about 550 mμ and that the reagent blank is negligible at this point. The other tertiary amine anesthetic complexes also showed maximum absorption at about this same wave length. Hence the same wave length setting or colorimeter filter may be employed regardless of which of these compounds is being assayed.

Range of anesthetic concentration.—The method applies over slightly different ranges of anesthetic concentrations, depending upon the individual anesthetic involved. The respective limits of concentration are listed in Table 1.

Within the ranges noted, the color intensity, as measured by colorimeter readings, showed an excellent straight-line relationship when plotted versus the respective anesthetic concentrations. Since some of the anesthetic bases do not remain stable in toluene solution for extended periods of time, it is suggested that fresh standard solutions be prepared daily.

Reaction with non-tertiary amines.—Various anesthetic compounds which do not contain a tertiary amine were subjected to the assay procedure. These agents included butethamine, ethyl aminobenzoate, meprylcaine, metabutethamine, and naepaine, in either their free base form or as their hydrochloride salts. In each case the colorimeter readings of the final solutions were negligible. When each of these agents was mixed with individual procaine solutions of known concentration, no interference whatsoever was observed when the procaine content was determined. This was the case even when the non-tertiary amine was present in tenfold concentration. It has been postulated[3] that primary and secondary amines do not enter into the color-producing reaction because of prior amide formation. Such amines, of course, might be acylated either by a portion of the *cis*-aconitic anhydride or by the acetic anhydride which is also present.

Analysis of procaine hydrochloride–butethamine hydrochloride mixtures.—A series of procaine hydrochloride–butethamine hydrochloride mixed solutions was prepared, using exact quantities of each anesthetic, in which the respective concentrations were varied. The solutions were then assayed for their procaine content. The purpose of this experiment was to ascertain the precision and accuracy of the procaine determination and to demonstrate that the presence of butethamine had no adverse effect on the procaine determination. As seen in Table 2, a high degree of reliability is associated with the procedure, and no interference due to butethamine was noted—even when the butethamine hydrochloride was included in many times the concentration present in the commercial mixture.

Analysis of the commercial mixture.—As a conclusive test of the usefulness of the method in the analysis of commercial mixtures of the anesthetics, a sample solution was prepared containing exact quantities of each active ingredient and excipient contained in the marketed drug and in the same proportions as indicated on the label. Samples of 1 ml. each of this solution were run through the extraction and assay procedure. The results compared most favorably with those noted in the previous section. This study served to confirm that even the contents of a single cartridge might be analyzed with confidence and that the other ingredients, such as epinephrine, sodium bisulfite, parabens, and sodium chloride, will not interfere.

Several samples of the marketed dosage form which were assayed by the procedure also yielded analytical results which were as close to theoretical values as pharmaceutical manufacturing processes will allow.

SUMMARY

The *cis*-aconitic anhydride method has been extended to the determination of other local anesthetics of dental interest which contain a tertiary amino group. Anesthetic compounds which do not contain a tertiary amine have been shown to give no interference with the method. The procedure has been found to have particular application in the analysis of commercial dosage forms which contain both procaine hydrochloride and butethamine hydrochloride in mixed solution.

TABLE 2

ANALYSIS OF KNOWN PROCAINE HYDROCHLORIDE–BUTETHAMINE HYDROCHLORIDE MIXTURES FOR PROCAINE HYDROCHLORIDE

Sample	Procaine Hydrochloride Actual (μM)	Butethamine Hydrochloride Actual (μM)	Procaine Hydrochloride Found (μM)	Procaine Hydrochloride, Per Cent of Theory*
1	0.100	0.100	0.100	100.0
2	0.100	0.100	0.098	98.0
3	0.100	0.500	0.099	99.0
4	0.100	0.500	0.100	100.0
5	0.100	1.000	0.102	102.0
6	0.100	1.000	0.099	99.0
7	0.500	0.500	0.498	99.6
8	0.500	0.500	0.503	100.6
9	1.000	1.000	1.000	100.0
10	1.000	1.000	1.003	100.3
11	1.000	5.000	0.992	99.2
12	1.000	5.000	0.989	98.9
13	1.000	10.000	1.003	100.3
14	1.000	10.000	0.998	99.8

* Precision 1.0 per cent.

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Acid Production in Sucrose by Oral Bacteria as Means of Estimating Caries Activity (Rickles Test)

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Acid production in glucose or sucrose by the bacteria in saliva has been described by several authors [1–4] as a means of estimating caries activity. These tests are all alike, in that a specimen of saliva and the carbohydrate of choice are incubated 4 hours, after which the amount of acid formed is measured. They differ only in the volume and concentration of the specimen or substrate and the method of measurement. Thus Fosdick, Hansen, and Epple[1] determined the amount (milligrams) of powdered enamel dissolved by the acid; Dewar[2] measured the pH change in the same test; Wach, Kesel, Hine, and O'Donnell[3] titrated the acid directly with standard alkali; and Rickles[4] utilized pH changes in a double indicator system. Acid formation in sucrose as described by Rickles[4] was selected as representative of these procedures for inclusion in a longitudinal program for the evaluation of laboratory tests, using saliva as the test material, for prediction of clinical change. This choice was made on the grounds of (*a*) the reported high correlation between in vitro reactions and in vivo clinical change occurring in a year and (*b*) ease of routine performance. The present paper deals with the results obtained by us in a study essentially duplicating that of Rickles, with the exception of age and sex of the subjects. Clinical correlations for *Lactobacillus* counts[5] and Snyder test reactions[6] recorded from the respective specimens of saliva are also included.

MATERIALS AND METHODS

A. *Clinical.*—The children in the longitudinal study of which this is a part were located in the Washington State School for the Deaf, Vancouver, Washington. Residence was limited to the school year. The children were examined clinically every 6 months, and specimens of saliva were collected monthly or semimonthly during the school year for 3 calendar years. It was easy, therefore, to introduce a given test at any scheduled time, conduct it for a given period, and obtain an assessment of its value in terms of clinical change occurring in the same or subsequent 6-month period. Of the 175 children in the study group from September, 1954, to September, 1955, only 138 were included in the evaluation of the Rickles test. These children (85 boys and 54 girls) ranged from five years and eight months to sixteen years and eleven months, with a mean age of eleven years and four months. The other 37 children were excluded on the following grounds: (*a*) not being present for the two clinical examinations on

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which the study was based and (*b*) not having a specimen of saliva collected within 60 days of the first clinical examination used as the base line. Clinical examinations were routinely performed by one examiner, using mouth mirror and explorer, who dictated his observations to a recorder. Bite-wing roentgenograms were taken by a technician. The films were developed each night for recall of faulty "takes." The same dentist examined the films and read his findings to the same recorder, who prepared all records, summarized the results, and transcribed these data to IBM punch cards for listing and analysis.

Measurement of clinical change for correlative purposes was made as follows: new carious surfaces, irrespective of dentition, were considered to be those which changed from sound to carious, doubtful to carious, and not present to erupted and carious, from the September, 1954, clinic to the September, 1955, clinic. Other possibilities, such as sound surfaces to filled and sound to filled and carious, were not included on the grounds of insufficient information. In any case the very small numbers falling in these categories were of no significance in the final analysis. Although the relative increment to decay (R.I.D.), an index for measuring clinical change irrespective of dentition, was computed for each individual for this period,[7] these figures are not given in this paper. These will be reported as part of a longitudinal study in which the entire clinical measurement is in terms of this index.

B. *Laboratory.*—Specimens of saliva were routinely collected at monthly intervals as a part of the longitudinal program. Since it was impossible to obtain pre-breakfast specimens, collection was made at a relatively constant time, 1–3 hours after breakfast, for each classroom by having each child chew a paraffin pellet 3–5 minutes and expectorate the stimulated saliva in a labeled bottle. The specimens were placed in an insulated box previously cooled to 5° C. with frozen plastic gel. The temperature of the box did not exceed 10° C. before it reached the laboratory. Placement of the saliva in sucrose solutions was completed within 4 hours after arrival of the test material. In view of the requirements for completion of the other laboratory tests, it was not deemed practicable to store these 48 hours or more, as were those used by Rickles, although it was later found in a small number of specimens (47), there was no difference between the pH changes obtained with freshly collected post-breakfast specimens and pre-breakfast samples held at room temperature for 48 hours. All laboratory tests were completed within 24 hours of collection, insofar as use of the saliva was concerned.

Preliminary testing was done exactly as described by Rickles in respect to preparation of reagents, indicator standards, volumes employed, size of tubes, temperature, and time of reading. Two difficulties were encountered: (*a*) an excessive amount of sediment was formed, which obscured comparison with color standards, and (*b*) the 13 × 100-mm. or Wassermann tube was awkward to handle where large numbers of tubes were to be inverted and allowed to settle before reading. To meet the first objection a small amount of agar (0.2 per cent) was added to the sucrose solution. This gel was sufficiently fluid to allow ready mixing of the saliva, yet viscous enough to prevent objectionable sedimentation. The resultant color changes in this gel permitted more accurate comparison with the standards than in the straight sucrose solutions, and the rate of acid formation was accelerated.

To overcome the problem of inversion, resort was made to larger tubes (16 × 150

mm.) which could be easily twirled or shaken instead of inverted. However, it was preferable to use larger volumes with the same ratio of components in the larger than in the smaller tubes. The final volume was 2.0 ml., of which 1.0 ml. was saliva and 1.0 ml. was 8 per cent sucrose containing the indicators, 0.004 per cent each of brom cresol green and brom cresol purple. No difference was noted in pH changes per hour in these than in the Wassermann tubes. Similarly, no difference could be observed in the reactions induced in substrates of commercial cane sugar or sucrose of analytical grade purity. It was also found unnecessary to sterilize the 8 per cent sucrose, since freshly prepared tubes could be stored a week or more in the refrigerator and show no sign of change when used as controls in the same series with sterile sucrose. This can probably be explained by the inhibitory action of this concentration of sucrose, the short time of incubation, and a small volume of substrate inoculated with massive numbers of bacteria in various phases of growth, effectively overwhelming any complicating action of the dormant bacterial cells in the unsterilized sucrose.

Therefore, all tests performed in this study were done as follows: unsterilized 1.0-ml. volumes of 8 per cent cane sugar containing the double indicator system, with and without 0.2 per cent agar, were inoculated with 1.0-ml. volumes of saliva, and these mixtures were incubated at 37° C. in a water bath. Reactions were observed at hourly intervals to record rate of change, but the limit was 4 hours, as recommended for this and similar tests. Color comparison with standards was best made by reflected light with the tubes slanted over a white background. Instrument colorimetry could not be done because of the turbidity introduced by the saliva and agar. Changes estimated to be halfway between the 0.4 increments of pH change were not recorded as such but as the next higher value: e.g., pH 6.0 as pH 6.2, pH 5.6 as pH 5.8, etc. For statistical purposes, the hourly readings were recorded in terms of pH and coded for transcription to IBM punch cards.

C. *Correlations.*—To obtain prediction in the manner of Rickles, the laboratory data were restricted to the values obtained from the first specimens of saliva collected within 60 days of the September, 1954, clinical examination. These figures were correlated with the number of new carious surfaces occurring in the interval from September, 1954, to September, 1955. Thus the actual lapse of time from the single sampling to the second clinical examination was 10–11½ months. It was impractical under our schedule to duplicate the 12–14-month interval listed by Rickles. The systematic collection of specimens of saliva also allowed correlation of the means of the laboratory data for the 6-month period, September, 1954, to March, 1955. These results were expressed as correlation coefficients (r).[8] Similar comparisons were made for *Lactobacillus* counts and Snyder test reactions from the respective specimens of saliva.

RESULTS

A. *Clinical measurements.*—Analysis of the clinical data showed the distribution of new caries experience in this group of children to be that given in Table 1.

This frequency chart shows an average of 4.2 new carious surfaces per child for 138 children living in this institution during the school year and at home for the balance. The only comparable figures for this area are those by Nevitt, Witter, and Bowman,[9] from whose data it was computed that, in 588 Milwaukie and Oregon City, Oregon, children from nine to fourteen years of age attending local schools, there was an average

of 4.7 new carious and filled surfaces per child from December, 1955, to April, 1957. So it would seem that the average caries experience in the Deaf School was no greater than that for children living at their homes within 20 miles of Vancouver, Washington.

B. *Laboratory.*—Although the primary correlation was to be based on the results of a single sampling of the children, the Rickles test was conducted on all the specimens of saliva collected during the 6-month period, September, 1954, to March, 1955. It was possible, therefore, to determine the validity of the single sampling in terms of the whole. This comparison of distribution of hourly pH changes in 8 per cent sucrose solutions with and without 0.2 per cent agar for the first set of specimens and the total collected is given in Tables 2 and 3. From these tables it can be observed that there

TABLE 1

FREQUENCY OF NEW CARIOUS SURFACES OCCURRING DURING A 12-MONTH PERIOD IN 138 CHILDREN GROUPED BY AGE

AGE	No. CHILDREN	No. NEW LESIONS											
		0	1	2	3	4	5	6	7	8	9	10	11–17
5–6	8	.....	1	.....	1	2	.....	1	.....	1	1	.....	1 (13)
7	11	.....	1	.....	2	1	2	3	.....	1	1	.....	.....
8	10	.....	.....	2	1	1	.....	1	2	2	.....	.....	1 (14)
9	9	2	1	2	1	.....	1	.....	.....	1	1	.....	.....
10	19	3	4	4	2	4	1	1	.....	.....	.....	.....	.....
11	21	3	4	2	3	3	1	1	3	1	.....	.....	.....
12	10	.....	1	.....	2	1	1	2	.....	1	1	.....	1 (15)
13	14	2	1	1	2	2	2	1	1	.....	1	.....	1 (17)
14	15	1	.....	1	2	1	4	.....	3	3	.....	.....	.....
15	11	.....	3	1	.....	3	1	1	1	1	.....	.....	.....
16	6	0	1	1	2	.....	1	1	.....	.....	.....	.....	.....
17–18	4	1	1	.....	1	.....	1	.....	.....	.....	.....	.....	.....
Total no. subjects	138	12	18	14	19	18	15	12	10	11	5	0	4
Total no. new lesions	584	0	18	28	57	72	75	72	70	88	45	0	59
Average	584/138=4.2 new carious surfaces/child/year												

was really very little difference in the distribution in any category, provided that one lumped the reactions of pH 5.0 and lower for each hour and group: e.g., 24 versus 19 per cent for sucrose at 4 hours and 67 versus 70 per cent for sucrose with agar at 4 hours. Therefore, we considered that the initial collection of saliva specimens was a valid sample of the whole. The effect of agar on acceleration of acid formation was clearly shown. In general, it advanced the readings by a third instead of the half originally thought to occur; that is, the same reactions appeared in 2 and a fraction hours in the presence of agar as compared with 4 hours in the sucrose alone. The precise role of the agar was not determined. It was thought to act by dispersing the clumps of bacteria in the gel so that the acid formation was widespread compared with the prompt sedimentation of the inoculum in the liquid medium and slower diffusion of acid.

C. *Correlations between laboratory and clinical data.*—1. Rickles Test.—The recorded pH changes induced by the initial or single set of specimens of saliva in 8 per cent sucrose broth after 4 hours' incubation, as well as those for the 2- and 4-hour incubations of sucrose-agar-saliva mixtures, were tabulated for comparison and correlation of the statistical means of clinical change expressed as new carious surfaces observed during a 12-month period or 10–11 months after sampling. This relationship with clinical change is illustrated in Figure 1, in which the almost linear curve obtained by Rickles is inserted for comparison.

As easily observed in Figure 1, there is little similarity between the correlations obtained by Rickles and by us. Based on the results of our study, it would have been im-

TABLE 2

HOURLY pH CHANGE IN 8 PER CENT SUCROSE BY FIRST SERIES OF SALIVA SPECIMENS FROM 138 CHILDREN COMPARED WITH REACTIONS INDUCED BY TOTAL NUMBER

pH	pH CHANGES (HOURS) (IN PER CENT)							
	1		2		3		4	
	S*	T*	S	T	S	T	S	T
6.6	97	93	68	63	39	33	19	16
6.2	0	5	18	20	24	23	15	14
5.8	1	1	9	10	14	17	13	16
5.4	1	1	4	6	17	23	29	35
5.0	1	0	1	1	6	4	23	19
4.6	.....	.....	.....	.....	.....	.....	1	0
4.2	.....	.....	.....	.....	.....	.....	.....	.....

* S = 138 specimens; T = 1,076 specimens.

TABLE 3

HOURLY pH CHANGE IN 8 PER CENT SUCROSE CONTAINING 0.2 PER CENT AGAR BY FIRST SERIES OF SALIVA SPECIMENS COLLECTED FROM 138 CHILDREN COMPARED WITH REACTIONS INDUCED BY TOTAL NUMBER

pH	pH CHANGES (HOURS) (IN PER CENT)							
	1		2		3		4	
	S*	T*	S	T	S	T	S	T
6.6	76	77	43	34	14	15	8	8
6.2	13	10	16	14	14	9	2	5
5.8	4	7	11	14	11	11	10	5
5.4	5	5	11	20	19	18	13	12
5.0	1	1	17	16	37	43	38	54
4.6	1	0	2	1	5	4	28	16
4.2	.....	.....	.....	.....	.....	.....	1	0

* S = 138 specimens; T = 1,076 specimens.

possible to predict with any degree of accuracy the number of new carious surfaces that actually occurred in any of the 138 children 10–11 months after sampling. This applied both to the original method (curve *B*) and our modification (curves *C* and *D*). The explanation lies in the minimal-maximal numbers of lesions for each laboratory unit. With few exceptions and these tempered by relatively few subjects in each, the ranges were too wide to allow any effective estimate of change. Since this test is one of acidogenesis without much selective inhibition or oral types and thereby somewhat simulates in vivo conditions, these results are of considerable interest in light of the accepted role of acidogenic bacteria in the carious process. It is more than likely that the large numbers of *Streptococcus salivarius* derived from the tongue and buccal mucous membranes, as shown by Krasse,[10] during the chewing of paraffin pellets accounts for some of this uniformity.

If, however, the respective means of the specimens collected during the 6-month period for comparative purposes are used for correlation, there is an apparent improvement toward the desired linearity for predictive purposes, as shown in Figure 2.

Again there was little similarity between our results with the Rickles method (curve

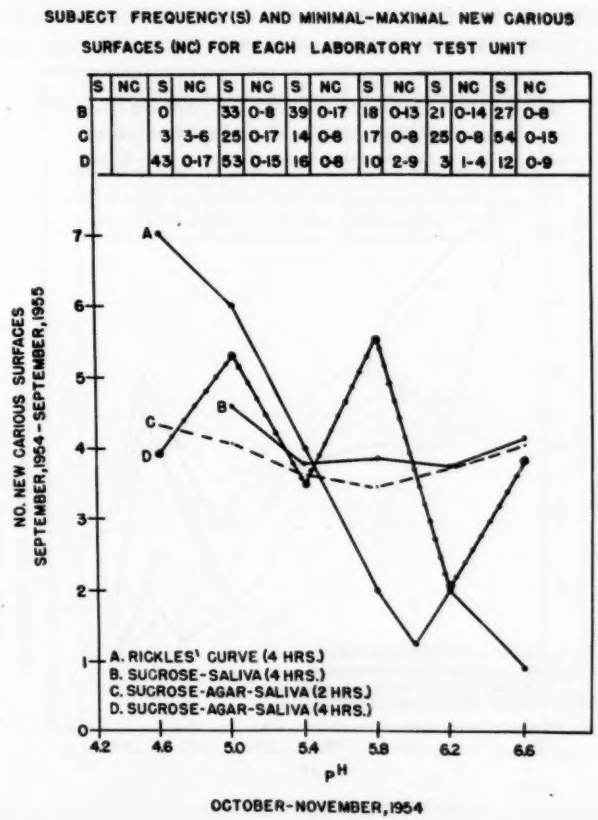
SUBJECT FREQUENCY(S) AND MINIMAL-MAXIMAL NEW CARIOUS SURFACES (NC) FOR EACH LABORATORY TEST UNIT
S NC S NC S NC S NC S NC S NC S NC
B 0 33 0-8 39 0-17 18 0-13 21 0-14 27 0-8
C 3 3-6 25 0-17 14 0-8 17 0-8 25 0-8 54 0-15
D 43 0-17 53 0-15 16 0-8 10 2-9 3 1-4 12 0-9
NO. NEW CARIOUS SURFACES SEPTEMBER, 1954 - SEPTEMBER, 1955
A
B
C
D
A. RICKLES' CURVE (4 HRS.)
B. SUCROSE-SALIVA (4 HRS.)
C. SUCROSE-AGAR-SALIVA (2 HRS.)
D. SUCROSE-AGAR-SALIVA (4 HRS.)
pH
OCTOBER-NOVEMBER, 1954

FIG. 1.—Comparison of pH changes in 8 per cent sucrose by single specimens of saliva with number of new carious surfaces observed 10–11 months later in 138 children.

B) and those reported by him. The improvement in respect to linearity was obtained with the agar modification and especially with the 4-hour period, but the steep rises in the last pH increments for curves *C* and *D* were limited to so few cases that reliability was considerably lessened. Whether these changes resulted from the mean being a better figure than a single one or the shortening of the period for estimation—6 instead of 10–11 months—is not clear, but it was probably the latter, since it has been shown that the initial sampling was a valid representative of the whole. On the other hand, the wide ranges of new surfaces for each pH unit prevented accurate prediction on an individual basis.

2. *Lactobacillus* Counts.—In performing *Lactobacillus* counts, all specimens of saliva in this study were diluted 1–10 in distilled water or physiological saline, and 0.1 ml. of this dilution was spread over the surfaces of plates of tomato-juice agar (pH 5.0) containing 1–20,000 sodium azide. Counts were recorded after 4 days, incubation as arithmetical figures brought to the nearest significant 5 and transposed to three-place logarithms for IBM recording. This method of recording leads to peculiar mathematical situations: viz., a logarithm of 0, means which are not arithmetical, and

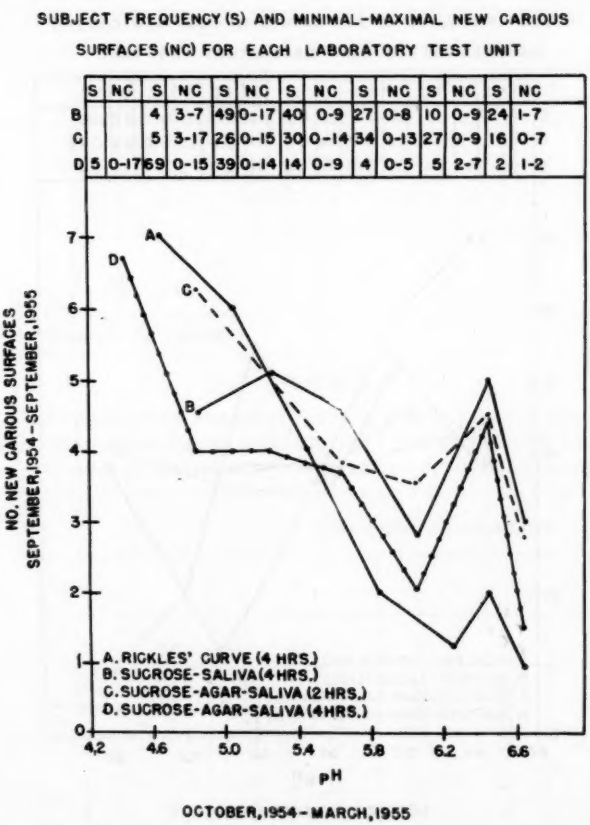
SUBJECT FREQUENCY (S) AND MINIMAL-MAXIMAL NEW CARIOUS SURFACES (NC) FOR EACH LABORATORY TEST UNIT
S NC S NC S NC S NC S NC S NC S NC
B 4 3-7 49 0-17 40 0-9 27 0-8 10 0-9 24 1-7
C 5 3-17 26 0-15 30 0-14 34 0-13 27 0-9 16 0-7
D 5 0-17 69 0-15 39 0-14 14 0-9 4 0-5 5 2-7 2 1-2
NO. NEW CARIOUS SURFACES SEPTEMBER, 1954 - SEPTEMBER, 1955
A
B
C
D
A. RICKLES' CURVE (4 HRS.)
B. SUCROSE-SALIVA (4 HRS.)
C. SUCROSE-AGAR-SALIVA (2 HRS.)
D. SUCROSE-AGAR-SALIVA (4 HRS.)
pH
OCTOBER, 1954 - MARCH, 1955

FIG. 2.—Comparison of mean pH changes in 8 per cent sucrose by specimens of saliva collected in a 6-month period with the number of new carious surfaces observed in 138 children in 12 months.

a gap from 0 to 2, since our negative counts must be interpreted as less than 100 lactobacilli/ml saliva. On the other hand, consistency allows comparison and increasing logarithmic values reflect higher *Lactobacillus* counts. The comparison of single *Lactobacillus* counts from the same specimens as those used for the Rickles tests and the respective means of the logarithmic values with clinical changes is given in Figure 3.

In Figure 3 it is clear that the distribution of counts for the single sampling is not too similar to those of the whole group (means), although some of the discrepancies may be attributed to such things as the method of grouping the two sets of data; that is, with the single specimens there were many more negative counts than in the means. The means values show the generally accepted relationship of increasing numbers of lactobacilli with larger numbers of new carious surfaces, but they allow no individual prediction because of the wide clinical range for each logarithmic value, which is essentially the same as reported by Glass.[11] This is particularly striking for the single sampling, where roughly one-third of the specimens were *Lactobacillus*-negative or contained less than 100 lactobacilli/ml of saliva, yet there was an average of 3.5 new surfaces per child in this group some 10–11 months later.

3. Acid Production in Brom Cresol Green Dextrose Agar (Snyder Test).—The rate of acid formation for each of 3 days' incubation in this highly selective medium by single specimens and the means of specimens of saliva (0.2 ml/test) collected during

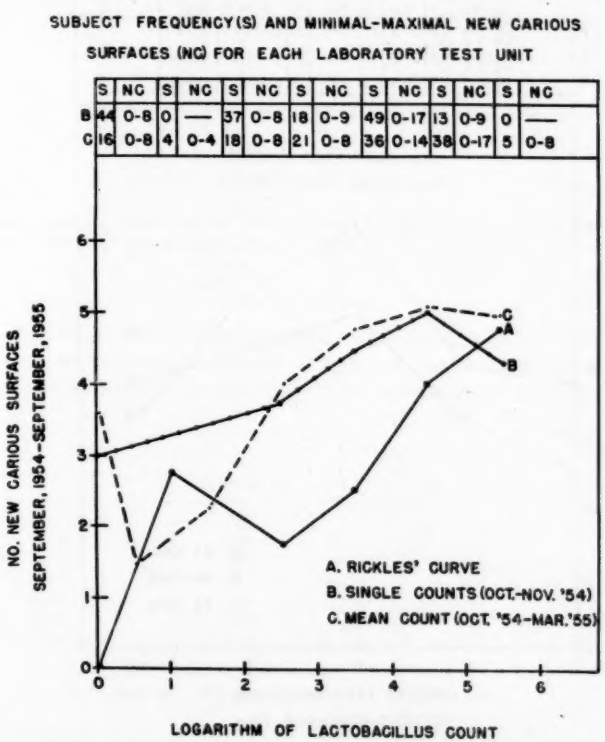
SUBJECT FREQUENCY(S) AND MINIMAL-MAXIMAL NEW CARIOUS SURFACES (NC) FOR EACH LABORATORY TEST UNIT
S NC S NC S NC S NC S NC S NC S NC
B 44 0-8 0 — 37 0-8 18 0-9 49 0-17 13 0-9 0 —
C 16 0-8 4 0-4 18 0-8 21 0-8 36 0-14 38 0-17 5 0-8
NO. NEW CARIOUS SURFACES SEPTEMBER, 1954-SEPTEMBER, 1955
LOGARITHM OF LACTOBACILLUS COUNT
A. RICKLES' CURVE
B. SINGLE COUNTS (OCT.-NOV. '54)
C. MEAN COUNT (OCT. '54-MAR. '55)

FIG. 3.—Comparison of single and mean *Lactobacillus* counts with number of new carious surfaces observed in 138 children.

this 6-month period were recorded in the usual 0–4 plus scale. Comparisons with the clinical changes observed in this group of children are shown in Figures 4 and 5.

The curves in Figures 4 and 5 show that increasing amounts of acid formed with longer incubation are generally associated with an increasing number of new carious surfaces, but the wide minimal-maximal ranges for each unit make individual prediction impossible. Since the Snyder test has been shown to be closely correlated with *Lactobacillus* count[12] and the latter has no individual predictability, similar conclusions for the Snyder test were to be expected. One thing is quite clear: these results do not support Snyder's assertion[13] that the rate of acid formation is significant or that positive reactions (2–4 plus) in 24 hours are indicative of greater caries activity than the same reactions in 48 or 72 hours or none at all for these periods.

The correlation coefficients for each of the laboratory tests with new caries experience and in turn those for the sucrose-saliva tests under evaluation were computed by standard methods.[8] These coefficients are listed in Table 4.

In statistical language the coefficients given in Table 4 may be interpreted as follows: The degree of correlation present between the different factors tested within these

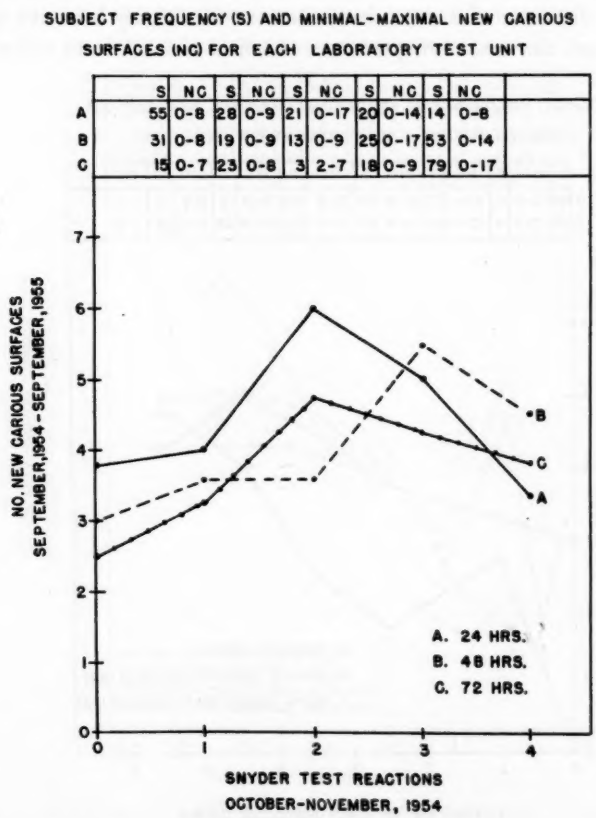
SUBJECT FREQUENCY (S) AND MINIMAL-MAXIMAL NEW CARIOUS SURFACES (NC) FOR EACH LABORATORY TEST UNIT
S NC S NC S NC S NC S NC
A 55 0-8 28 0-9 21 0-17 20 0-14 14 0-8
B 31 0-8 19 0-9 13 0-9 25 0-17 53 0-14
C 15 0-7 23 0-8 3 2-7 18 0-9 79 0-17
NO. NEW CARIOUS SURFACES SEPTEMBER, 1954 - SEPTEMBER, 1955
0
1
2
3
4
5
6
7
A. 24 HRS.
B. 48 HRS.
C. 72 HRS.
SNYDER TEST REACTIONS OCTOBER-NOVEMBER, 1954

FIG. 4.—Comparison of Snyder test reactions by single specimens of saliva with number of new carious surfaces observed 10–11 months later in 138 children.

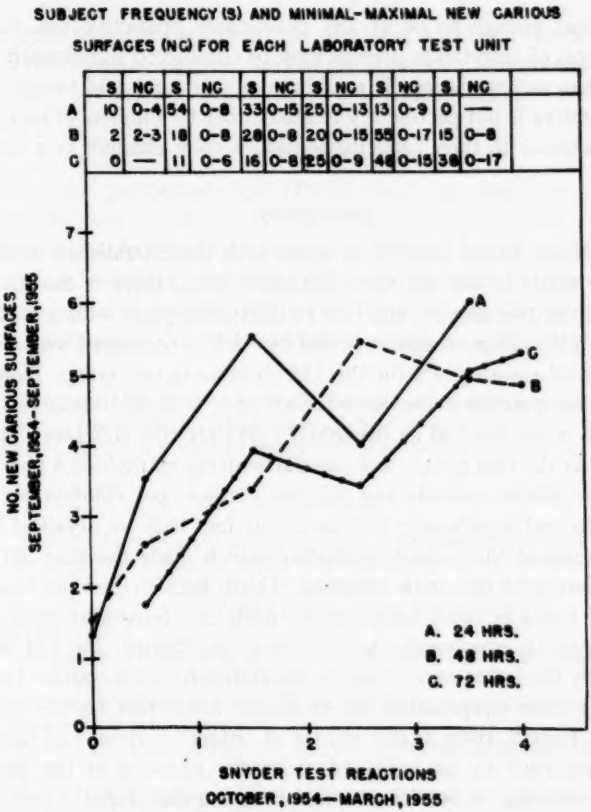
SUBJECT FREQUENCY (S) AND MINIMAL-MAXIMAL NEW CARIOUS SURFACES (NC) FOR EACH LABORATORY TEST UNIT
S NC S NC S NC S NC S NC S NC
A 10 0-4 54 0-8 33 0-15 25 0-13 13 0-9 0 —
B 2 2-3 18 0-8 28 0-8 20 0-15 55 0-17 15 0-8
C 0 — 11 0-6 16 0-8 25 0-9 46 0-15 38 0-17
NO. NEW CARIOUS SURFACES SEPTEMBER, 1954-SEPTEMBER, 1955
A. 24 HRS.
B. 48 HRS.
C. 72 HRS.
SNYDER TEST REACTIONS
OCTOBER, 1954—MARCH, 1955

FIG. 5.—Comparison of mean Snyder test reactions by specimens of saliva collected in a 6-month period with the number of new carious surfaces observed in 138 children in 12 months.

TABLE 4*

CORRELATION COEFFICIENTS (*r*-VALUES) FOR RICKLES TESTS WITH CLINICAL CHANGE AND OTHER LABORATORY TESTS

Y	*X*			
	Surfaces	R	R/A-2	R/A-4
R	0.189			
R/A-2	.252			
R/A-4	.109			
LBC	.311	0.224	0.247	0.229
S-24	.254	.330	.308	.362
S-48	.153	.253	.308	.366
S-72	0.323	0.268	0.252	0.346

* R = Rickles test; LBC = *Lactobacillus* count; S = Snyder test.

groups is not great enough to be of any practicable predictive use. In most cases, however, the degree of correlation present may be considered significantly greater than zero or greater than no correlation. It would also appear that acid formation in sucrose by specimens of saliva is not pertinently related either to numbers of lactobacilli in the respective specimens or to their capacity to induce color changes in a highly selective medium.

DISCUSSION

Since these findings do not support or agree with those published by Rickles, some attempt must be made to explain the differences. First, there is the discrepancy between the ages of the two groups: eighteen to thirty-five years with a mean of twenty-two years for the 135 college students studied by Rickles compared with six to eighteen years and a mean of eleven years for the 138 children in our group. However, on the basis that the caries process is the same for all ages, it should be as easy to measure in one group by a given method as the other. Certainly, the difference in the average caries experience in the two groups was not particularly marked: 3.4 new surfaces per individual for the college students and 4.2 new surfaces per child in our group. This seemed to have no real significance in view of the fact that we obtained better agreement with the means of the 6-month sampling, which made the time between testing (mean) shorter than with the single sampling. Third, Rickles used pre-breakfast specimens held 48–96 hours at room temperature, while our tests were done with freshly collected post-breakfast specimens. As previously mentioned, the pH changes in 8 per cent sucrose by the latter were found to be similar to those collected before breakfast and stored at room temperature for 48 hours; hence this feature seems to be of little importance. Fourth, there is this matter of "blind" matching of laboratory with clinical changes recorded by an independent dentist unaware of the purpose of the laboratory study, whereas in our longitudinal program the clinical examinations were performed by the same dentist with full knowledge of the project. However, laboratory and clinical data were kept separate until that part of the project was completed and then brought together, so that this study is just about as "blind" as the other. It is doubtful whether the assessment of clinical change by these dentists was of such order as to account for the differences in the two studies. Fifth, the groups were identical in minimal-maximal ranges (0–17) of clinical change, but only 8 of Rickles groups showed no clinical change 1 year later as compared with 12 in ours. The essential difference was in the distribution of caries experience, with particular reference to the clinically negative, who were so spread in our study that, for practically every laboratory test unit, the range was from zero to at least half the maximal number of new carious surfaces observed for any one case. Thus, in our correlations with acid production in sucrose alone (Rickles test), the range for number of lesions was 0–9 for the highest value, pH 6.6, and the same for the lowest, pH 5.0, with 0–17 surfaces for the intermediate units. The same relationships existed for the *Lactobacillus* counts and Snyder test reactions. Under these conditions, therefore, it would be obviously impossible to make even an educated guess from the laboratory data about the expectancy of a given number of lesions in an individual 1 year after sampling. Considering the fact that Rickles could get no closer than a range of three lesions for prediction

at the 68 per cent confidence level and we could not approach this accuracy, it would seem that acid formation in sucrose, *Lactobacillus* counts, or Snyder tests do not allow individual prediction in respect to number of new surfaces observed 6–12 months later.

SUMMARY

Acid formation in 8 per cent sucrose (Rickles test) by single specimens of saliva or specimens collected over a 6-month period from 138 children was shown to have so little correlation with number of new carious surfaces observed per child 6–12 months after sampling that individual predictability was impossible. Similar conclusions were reached for estimation of caries activity in this group by *Lactobacillus* counts and Snyder test reactions using the same specimens of saliva as test material.

We are deeply indebted to Dr. Donald R. Porter, Department of Pedodontia, for the clinical examinations and to Mrs. Fern R. Macho, Department of Bacteriology, for the recordings of all clinical data for IBM listings, and to Mr. Richard Bunch for the statistical analysis.

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Induction of Dental Caries and Pathological Changes in Periodontium of Rat with Hydrogen Peroxide and Other Oxidizing Agents

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In preliminary studies in this laboratory it was observed that immature rats fed a stock ration and administered hydrogen peroxide in the drinking water as a 1 or 1.5 per cent solution developed extensive carious lesions and pathological changes in the periodontium. These effects were noted as early as the third week of peroxide administration and were well advanced after an 8-week period. They did not occur, however, in rats fed a similar diet with hydrogen peroxide omitted from the drinking water. The present series of experiments was undertaken to confirm and extend these earlier observations.

MATERIALS AND METHODS

Experiment I. Effects of graded levels of hydrogen peroxide in the drinking water on the teeth and periodontium of rats.—Ninety-six male rats of the Holtzman strain were selected at an average body weight of 51.8 gm. (range 50–55 gm.) and were divided into four comparable groups of 24 animals each. The four groups were provided a stock ration* and drinking fluid ad libitum but differed as follows: group I received distilled water as the source of drinking fluid, whereas groups II, III, and IV were provided with distilled water to which H_2O_2 had been added in amounts sufficient to make a $\frac{1}{2}$, 1, and 1.5 per cent solution, respectively.† The rats were placed in metal cages with raised screen bottoms (2 animals per cage) and were weighed weekly during the course of the experiment. Surviving animals were sacrificed after 8 weeks. Their heads and submaxillary glands were removed and fixed immediately in an 80 per cent alcoholic Bouin's (picric-acetic-formalin) solution. The heads were dacalcified in 10 per cent nitric acid in 10 per cent formalin and washed with a saturated lithium carbonate solution. They were then dehydrated and infiltrated in the routine manner, imbedded in 50 per cent nitrocellulose, sectioned at 20 μ thickness, and stained with hematoxylin and eosin, Mallorys' anilin blue collagen stain, and periodic acid. The salivary glands were imbedded in paraffin, sectioned at 7 μ, and stained with sematoxylin and eosin.

Experiment II. Comparative effects of inanition and hydrogen peroxide administered in the drinking water on the teeth and periodontium of rats.—Fifty male rats of the

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* Wayne Lab Blox, Allied Mills, Inc., Chicago, Illinois.

† The pH of these solutions was 6.55, 6.42, and 6.30, respectively.

Holtzman strain were selected at an average body weight of 42.6 gm. (range 39–46 gm.) and were divided into five comparable groups of 10 animals each. All rats were fed a diet similar in composition to that employed in Experiment I. However, whereas the diet in Experiment I was in the form of hard pellets or blocks approximately $\frac{5}{8}$ inch wide, 1 inch long, and $\frac{3}{8}$ inch high, that administered in the present experiment was provided as a finely ground powder. Group A received this diet ad libitum; groups B and C were fed the diet at levels of 7.5 and 6 gm. per day, respectively. All three of the above groups were administered distilled water ad libitum. Groups D and E were fed ad libitum but were given hydrogen peroxide in the drinking water as a 1 and 1.5 per cent solution, respectively. Animals were placed in individual metal cages and were weighed weekly during the course of the experiment. Surviving animals were sacrificed after 8 weeks, and histologic sections were prepared of the teeth, periodontal tissues, and submaxillary glands, as indicated above.

Experiment III. Effects of dietary supplements of zinc peroxide, potassium permanganate, and sodium perborate on the teeth and periodontium of rats.—Subsequent to the experiments indicated above, preliminary studies were conducted on the effects of oxidizing agents other than hydrogen peroxide on the induction of dental caries and periodontal disease in the rat. Twenty-four male rats of the Holtzman strain were selected at an average body weight of 43.2 gm. (range 40–45 gm.) and divided into four comparable groups of 6 animals each. One group (group a) was fed the powdered diet employed in Experiment II; the others (groups b, c, and d) were fed the powdered diet supplementd with 2 per cent zinc peroxide, 2 per cent potassium permanganate, and 2 per cent sodium perborate, respectively. Distilled water was provided ad libitum to all groups. The experimental procedure was similar to that employed in the previous experiments. After 8 weeks of ad libitum feeding, animals were sacrificed, and histologic sections were prepared of the teeth and periodontal tissues.

RESULTS

Experiment I.—Growth was significantly retarded in all rats which received hydrogen peroxide in their drinking water, the degree of retardation being proportional to the peroxide concentration. At the time of sacrifice the average body weight of rats in the various groups was as follows: group I, 331.6 ± 6.9 gm.;* group II, 222.9 ± 15.0 g.m.; group III, 79.6 ± 4.8 gm.; and group IV, 59.6 ± 7.3 gm. Seven of the rats in group IV died during the course of the experiment; the remaining animals in this group and all those in the other groups survived. In agreement with preliminary findings, hydrogen peroxide when incorporated in the drinking water as a 1 or 1.5 per cent solution resulted in extensive carious lesions and pathological changes in the periodontium after an 8-week period of administration. All surviving animals in group IV and 15 of the 24 rats in group III showed such changes. Seven out of 24 rats in group II also had caries, but the latter were mild. No periodontal damage was observed in this group. The control rats in group I exhibited neither carious nor periodontal damage (Figs. 1, 2 and 7).

The carious lesions started at the tip of the cusp and extended through the dentin to the pulp (Figs. 3, 4). In the more severely affected animals, complete destruction of the

* Including standard error of the mean.

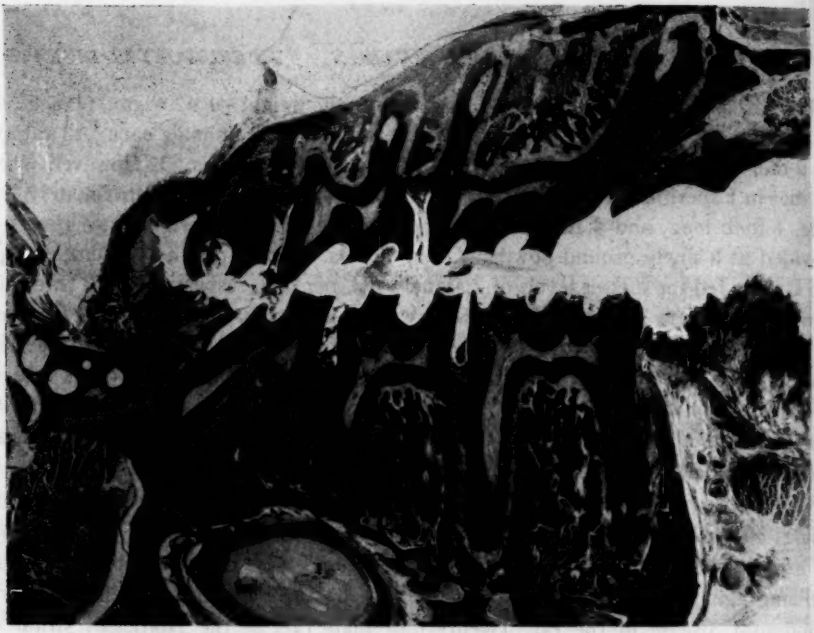

FIG. 1.—Section of jaws, control animal (group I). Mallory's stain. 20μ, $\times$10

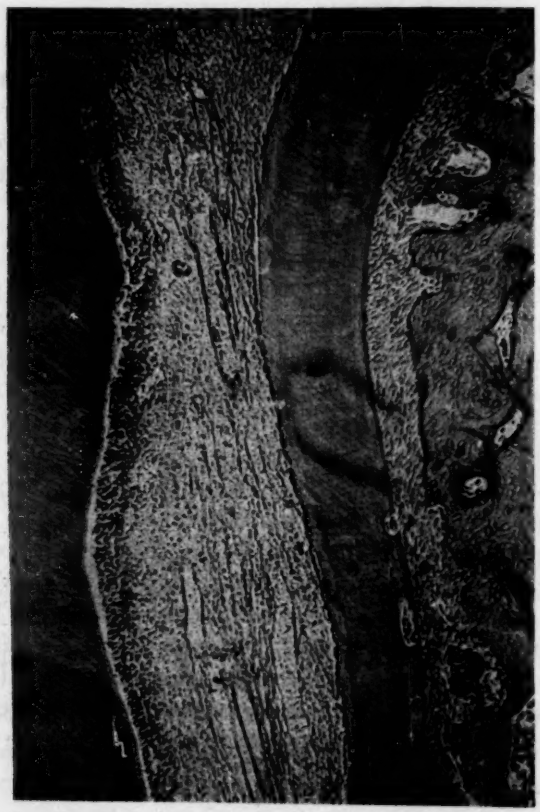

FIG. 2.—Section of molar distal root area of control animal (group I), showing dentin, pulp, periodontal ligament, and alveolar process. Note resorption area on root surface. Stain hematoxylin and eosin. 7μ, $\times$125.

FIG. 3.—Section of jaws of experimental animal receiving 1.5 per cent hydrogen peroxide in drinking water (group IV). Note caries in lower first molar and tips of cusps of upper molars. Note extent of pulp necrosis. Mallory's stain. 20μ, $\times$10.

FIG. 4.—Lower first molar of experimental animal receiving 1.5 per cent hydrogen peroxide in drinking water (group IV). Note pulp and periapical necrosis. Carious areas at the tip of cusp. Mallory's stain. 20μ, $\times$30.

crown and necrosis of the pulp, with fragmentation of the tooth, were observed. Caries occurred in the molars of both mandible and maxilla, being more prevalent in the lower jaw than in the upper. It was most marked in the first molar, although the second molars and to a lesser extent the third were also affected. Some of the damage to the periodontium appeared to be directly related to the caries (Figs. 3, 4), while other effects, such as resorption of cementum, dentin, and alveolar bone, gingival hyperplasia, and necrosis of the gingiva, appeared to be independent of the carious process. The periodontal changes varied in severity from gingival hyperplasia to necrosis of gingival papillae (Fig. 5). Resorption of alveolar crest cementum and dentin was also noted in some animals at the lingual margin of the upper incisor teeth (Fig. 6). It is of interest that small areas of cementum resorption, although less extensive than the above, also occurred on the distal aspect of the root surface of control rats (group I) (Fig. 2). These areas sometimes penetrated the dentin layer but were usually confined to the cementum.

Resorption of the lamina dura and supporting bone was frequent in peroxide-treated

FIG. 5.—Interdental gingival papilla in molar area of animal receiving 1.5 per cent hydrogen peroxide in drinking water (group IV). Note necrosis of interdental papilla. Mallory's stain. 7μ, ×30.

animals both in the interradicular space and on the mesial and distal root surfaces along the whole length of the root. Destruction of periodontal ligament fibers associated with inflammatory changes and ulceration was also noted. Early changes in the gingival margin (Fig. 8) were demonstrated as a loss of collagen fibrils with an infiltration of inflammatory cells, primarily plasma cells and lymphocytes. The pattern of arrangement of the collagen fiber bundles was altered in the experimental animals, with the free gingival fibers and alveolar crest fibers depleted or absent. The epithelium on the buccal aspect was edematous, with hyperkeratosis and parakeratosis. The pocket epithelium appeared unaffected.

Atrophic changes were observed in the secretory cells of the submaxillary glands of rats in groups III and IV (Fig. 10). These changes were not present in the rats of group I (Fig. 9) and were either absent or present to a minimal degree in rats of group II.

Experiment II.—In agreement with the earlier experiment, growth was significantly retarded in rats administered the 1 and 1.5 per cent hydrogen peroxide solutions

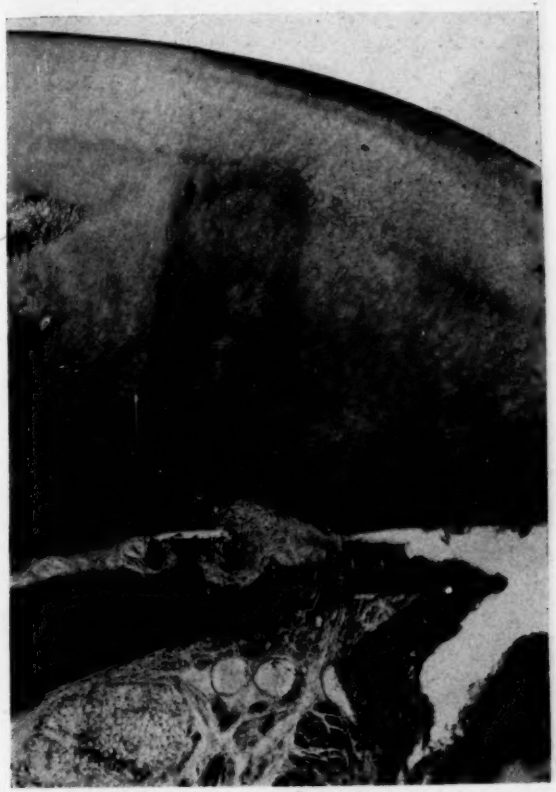

FIG. 6.—Upper incisor of animal receiving 1.5 per cent hydrogen peroxide in drinking water (group IV). Note resorption of lingual alveolar crest and of dentin. Also note destruction of periodontal ligament fibers. Mallory's stain. 20μ, $\times$35.

(groups D and E), although to a lesser extent than in the former experiment.* Growth was also retarded in rats fed the restricted diets. The average body weight of rats in the various groups at the time of sacrifice was as follows: group A, 304.0 ± 9.3 gm.; group B, 133.2 ± 1.6 gm.; group C, 106.5 ± 1.2 gm.; group D, 131.0 ± 5.4 gm.; and group E, 85.1 ± 4.5 gm. Only 1 of the 10 rats in group E died during the course of the experiment. All other rats in this and the other groups survived.

Histologically, no significant differences were observed between the rats given hydrogen peroxide in the present experiment (groups D and E) and the corresponding groups in Experiment I (groups III and IV). The incidence and severity of caries and periodontal damage were comparable in the two experiments. Dental caries and pathological

* The precise cause of the smaller weight increment of peroxide-treated animals in Experiment I is not known. It is possible that, as a result of the gingival changes in the peroxide-treated rats, the "hard" pelleted ration fed in Experiment I caused more trauma and pain than the powdered ration employed in Experiment II, with the result that food consumption (and gain in body weight) were less for the peroxide-treated rats in Experiment I.

FIG. 7.—Gingival margin of control animal (group I). Note normal arrangement and distribution of epithelium and connective-tissue fiber bundles of periodontal attachment apparatus. Mallory's stain. 20μ, ×30.

changes in the periodontium and submaxillary glands were not observed in rats fed the restricted diets (groups B and C). Group A was comparable in all respects to the normal controls (group I) of the former experiment.

Experiment III.—Growth was significantly retarded in all rats administered the zinc peroxide, potassium permanganate, or sodium perborate supplements. After 8 weeks of feeding, the average body weight of rats in the various groups was as follows: group a, 308.6 ± 8.6 gm.; group b, 174.0 ± 11.1 gm.; group c, 179.7 ± 8.4 gm.; and group d, 227.4 ± 12.1 gm. Dental caries with pulp necrosis similar to that obtained with hydrogen peroxide at a 1 or 1.5 per cent level in the drinking water was observed in the rats fed the zinc peroxide and sodium perborate supplements (groups b and d). In addition, rats fed the latter supplements exhibited marked resorption of the whole root surface, with only a thin dentinal layer remaining at the time of sacrifice (Fig. 11). Periodontal damage and caries with pulp necrosis did not occur in rats fed the potassium permanganate supplement (group c). One hundred per cent of the latter series, however, had surface caries.

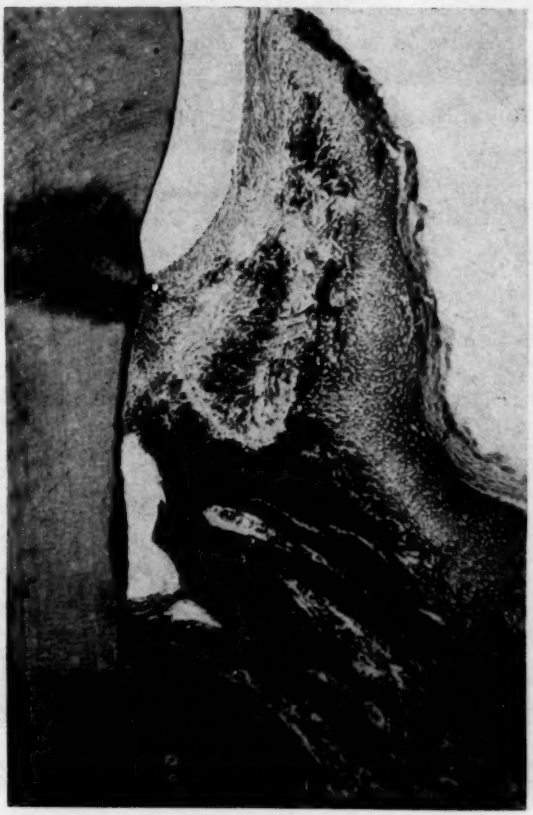

FIG. 8.—Gingival margin of animal receiving 1.5 per cent hydrogen peroxide in drinking water (group IV). Note edema, parakeratosis, and slight hyperkeratosis in epithelium. Note especially destruction of free gingival collagen fiber bundles. Mallory's stain. 20μ, ×30.

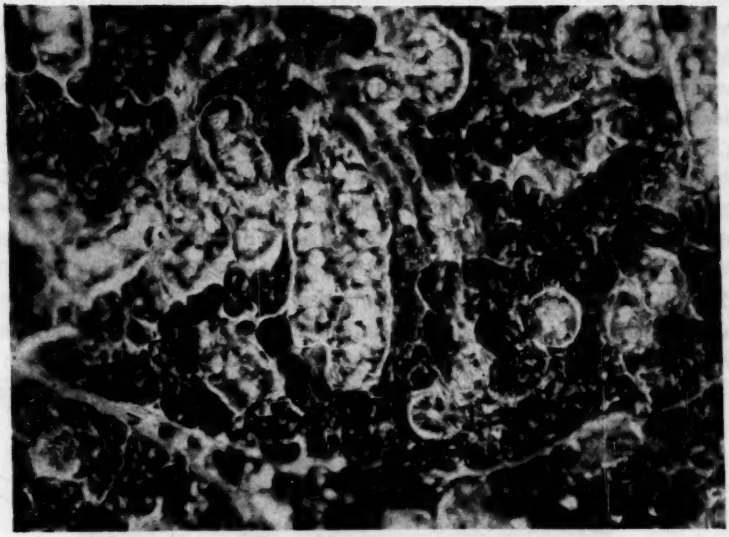

FIG. 9.—Submaxillary gland of control animal (group I). Stain hematoxylin and eosin. 7μ, $\times$250

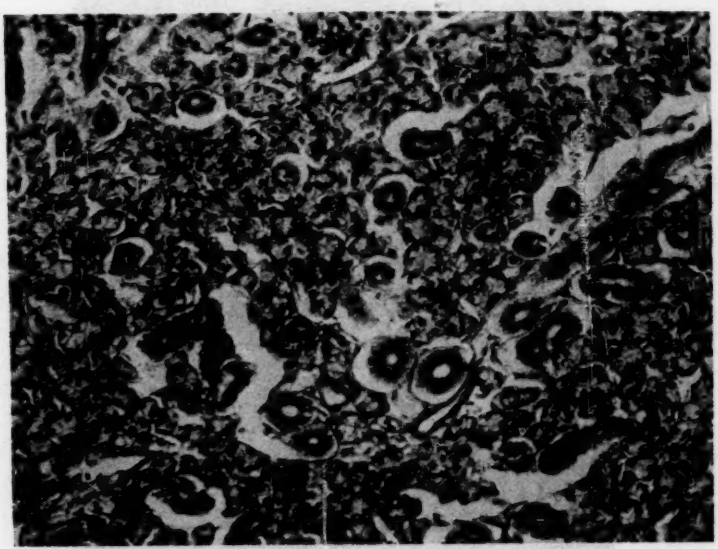

FIG. 10.—Submaxillary gland of animal receiving 1.5 per cent hydrogen peroxide in drinking water (group IV). Note pyknosis of all cellular elements. Stain hematoxylin and eosin. 7μ, $\times$250.

DISCUSSION

Present findings indicate that hydrogen peroxide when administered as a 1 or 1.5 per cent solution in the drinking water induces dental caries in the rat. The carious lesions start at the tip of the cusp and extend through the dentin to the pulp. In the more severely affected animals, complete destruction of the crown and necrosis of the pulp with fragmentation of the tooth occur. The type of caries induced differs significantly in origin and apppearance from the "occlusal," "fissure," and "smooth-surface" caries more commonly observed in the rat.[1-3] It is of interest that the caries occurred despite the fact that the rats were fed a "non-cariogenic" stock ration, on which caries were never observed in the absence of hydrogen peroxide treatment. This is the first report, so far as is known to the present investigators, in which caries have been induced in rats fed a stock ration adequate for normal growth and reproduction without the special dietary modifications required for caries production in the past.

It is felt that the production of caries on such a diet constitutes a potentially useful

FIG. 11.—Molar tooth of the animal receiving 2 per cent zinc peroxide in the food ration. Note dentin resorption along total outline of root, with destruction of periodontal ligament fibers, loss of interradicular bone, and necrosis of pulp. Mallory's stain. 7μ, $\times$30.

"tool" for the study of anticaries agents that is superior in a number of respects to the use of animals whose caries develop as a consequence of the administration of very specialized diets differing radically from that commonly ingested by man. Present findings also suggest the desirability of further studies on the possible role of oxidizing agents of bacterial, salivary, or food origin in the etiology of caries and periodontal disease in man.

Findings of the present experiments are not to be interpreted as indicating that hydrogen peroxide and other oxidizing agents as used clinically have a deleterious effect. The dosages employed and the length of time that the oxidizing agents were administered in the present experiments are never encountered under clinical conditions. On the contrary, there is some evidence that oxidizing agents at low levels in the diet may actually inhibit caries formation (as was reported by Hein, Wisotzky, and Quigley,[4] with a supplement of 0.3 per cent sodium carbonate peroxide in the diet) or have no effect at all (as was observed by Hein and Wisotzky[5] with low levels of potassium permanganate).

No data are available as to the mechanisms whereby hydrogen peroxide and the other oxidizing agents employed in the present experiment exert their deleterious effect. Changes in the oral flora induced by these materials may be implicated in the findings obtained. In view of the pathological changes induced by oxidizing agents in the submaxillary glands, it may be that resulting modifications in salivary flow or secretion contributed to the observed results. Goldhaber[6] recently observed that hyperoxia induced by the introduction of 95 per cent O_2 and 5 per cent CO_2 into the atmosphere of tissue cultures of young mouse calvaria consistently resulted in rapid and marked bone resorption. It is possible that the administration of hydrogen peroxide and other oxidizing agents in excessive amounts induces hyperoxia at the cellular level, which may in turn contribute to the findings noted.

SUMMARY

Immature rats fed a non-cariogenic ration and administered hydrogen peroxide in the drinking water as a 1 or 1.5 per cent solution developed extensive carious lesions and pathological changes in the periodontium and submaxillary glands after an 8-week period of administration. Similar effects were noted when zinc peroxide and sodium perborate were incorporated at a 2 per cent level in the ration. The significance of these findings is briefly discussed. These dental and periodontal changes appeared to be independent of weight loss brought about by inanition.

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Studies in the Design and Analysis of Dental Experiments

II. *A Four-Way Analysis of Variance*

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The analysis-of-variance technique, originally developed by Sir Ronald A. Fisher,[1] has proved of great utility in statistical analysis. Since its introduction, the technique has been extended in many directions. If many criteria of classification are involved, the methods of analysis, as well as the interpretations, become quite complicated.[2] In spite of these complications, the procedure has the great advantage of using one over-all statistical analysis to answer a series of questions from the data. It is the purpose of this paper to present, in detail, a method of calculation and the resulting interpretations for an analysis of variance in which four variables, i.e., four criteria of classification, are studied.

DESCRIPTION OF EXPERIMENT

A study was made of plasma salicylate levels obtained at four time periods (10, 15, 20, and 30 minutes) on 28 individuals after taking standard tablets of code drug B or code drug A.[3] After an overnight fasting period and following withdrawal of a control blood sample, each subject received two tablets of one of the drugs, which were swallowed without chewing, followed by 100 ml. of water. One half of the group was chosen at random to receive drug A, while the other half received drug B. After an interval of at least 3 days (to eliminate residual effects of prior medication) those individuals who had received drug A were then given drug B and vice versa. Some time later, the whole procedure was repeated, again in a random manner, to constitute a second run of the experiment. Since the individuals differed slightly in the readings of the original plasma control samples, these readings were used to correct those of the subsequent four time periods after taking the medication. In this manner, all readings correspond to equal initial readings. The data appear in Tables 1*a* and 1*b*.

In this experiment the plasma salicylate levels are completely cross-classified with respect to four criteria: individuals, drugs, periods (or time intervals), and runs. In this type of experimental model, the investigators are regarding three of the criteria or factors as "fixed": the two specific drugs; the four specific time periods after the subjects took the drug preparations; and the two runs of the experiment. The 28 individuals on whom the data were drawn constituted, however, a "random" sample of all

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individuals who could have been studied. The type of experimental model that we have may therefore be regarded as a "mixed model," i.e., neither a pure "fixed" nor a pure "random model." This type of model is a frequent one found in clinical experimentation, although there are not always four criteria. The model dictates the methods of statistical analysis to be employed and the interpretations to be made therefrom.

CALCULATIONS

The most convenient method for calculating the various terms of the analysis of variance with four criteria would be to (*a*) analyze the data for each run as a three-way analysis of variance;[4] (*b*) perform a three-way analysis of variance on the sum of the measurements of runs 1 and 2 to obtain the terms of the four-way analysis of variance not involving runs; and (*c*) subtract the terms under *b* from the sums of the corresponding terms in *a*, to obtain the terms of the four-way analysis of variance involving run.

TABLE 1*a*

PLASMA SALICYLATE LEVELS (MG. PER CENT) FOR DRUG B AND DRUG A, RUN 1

IND.	DRUG B					DRUG A				
	10 min.	15 min.	20 min.	30 min.	Ind. Total	10 min.	15 min.	20 min.	30 min.	Ind. Total
1	0.15	0.25	1.35	2.35	4.10	0.75	1.05	1.70	3.10	6.60
2	1.20	2.20	3.30	4.25	10.95	0.10	0.15	0.50	0.75	1.50
3	0.30	1.50	1.85	2.70	6.35	0.30	0.80	1.30	2.20	4.60
4	0.50	1.60	3.15	3.70	8.95	0.65	1.35	1.75	2.35	6.10
5	1.20	1.40	1.70	3.35	7.65	0.30	0.55	1.10	1.30	3.25
6	0.65	2.50	5.00	5.60	13.75	0.80	1.70	1.80	2.80	7.10
7	0.40	1.15	1.40	1.60	4.55	0.40	0.60	0.75	1.55	3.30
8	0.90	2.40	3.75	5.00	12.05	1.25	2.50	2.90	4.15	10.80
9	1.00	2.00	3.75	4.80	11.55	0.70	1.05	2.00	3.70	7.45
10	1.40	3.30	3.50	5.85	14.05	0.10	0.18	0.85	1.10	2.23
11	0.45	1.15	2.35	4.05	8.00	0.40	0.60	0.83	1.30	3.13
12	1.15	4.15	6.05	6.95	18.30	0.50	0.75	0.95	2.50	4.70
13	1.08	1.10	2.15	2.34	6.67	0.25	0.55	0.80	1.15	2.75
14	1.60	2.75	3.56	5.10	13.01	0.45	0.55	0.95	1.35	3.30
15	0.20	0.40	0.95	1.15	2.70	0.35	0.65	1.55	2.05	4.60
16	0.40	0.42	1.95	2.40	5.17	0.70	0.80	1.45	2.80	5.75
17	0.60	0.65	1.50	3.70	6.45	0.10	0.25	0.30	0.80	1.45
18	2.40	4.10	4.45	6.15	17.10	0.45	0.55	0.85	1.00	2.85
19	0.90	3.00	3.10	4.00	11.00	0.00	0.35	0.70	1.15	2.20
20	2.00	3.45	4.35	6.05	15.85	0.25	1.90	2.18	3.70	8.03
21	0.65	0.80	1.15	2.40	5.00	0.55	0.75	0.95	1.45	3.70
22	1.45	2.95	3.95	4.85	13.20	0.70	1.20	1.62	1.95	5.47
23	1.70	2.35	2.65	5.05	11.75	0.65	1.10	1.65	2.35	5.75
24	2.35	3.20	4.15	4.45	14.15	1.10	3.25	3.60	4.77	12.72
25	1.65	3.40	3.50	3.60	12.15	0.60	1.10	1.30	2.25	5.25
26	0.20	0.55	1.70	3.45	5.90	0.20	0.25	0.80	1.70	2.95
27	0.00	0.45	1.50	2.70	4.65	0.10	1.00	1.20	1.30	3.60
28	1.70	1.85	2.10	3.35	9.00	0.60	0.65	1.40	1.75	4.40
Period total	28.18	55.02	79.86	110.94	274.00	13.30	26.18	37.73	58.32	135.53

Three-way analysis of variance on each run.—The first step is to obtain the component sums of squares for the sources of variation for each run of the experiment. These seven sources of variation are as follows: between individuals, between periods,

between drugs, individual-period interaction, individual-drug interaction, period-drug interaction, and individual-period-drug interaction (or triple interaction).

To obtain these sums of squares (SS) for the first run, we need the individual totals specific for drug given in Table 1*a*. We also need a summary table in which the values for each individual are added for drug B and drug A at each of the four time periods. Thus, for the first individual, $0.75 + 0.15 = 0.90$; $1.05 + 0.25 = 1.30$; $1.70 + 1.35 = 3.05$; $3.10 + 2.35 = 5.45$, and similarly for the 27 other individuals. A portion of this summary table appears as Table 2.

TABLE 1*b*

PLASMA SALICYLATE LEVELS (MG. PER CENT) FOR DRUG B AND DRUG A, RUN 2

IND.	DRUG B					DRUG A				
	10 min.	15 min.	20 min.	30 min.	Ind. Total	10 min.	15 min.	20 min.	30 min.	Ind. Total
1	0.65	1.95	2.30	4.40	9.30	1.00	1.12	2.00	3.60	7.72
2	0.40	0.45	0.60	0.70	2.15	0.00	0.15	0.90	1.40	2.45
3	1.00	1.60	2.30	2.60	8.00	0.45	0.65	1.13	2.25	4.48
4	1.00	1.60	2.25	2.30	7.15	0.55	0.70	1.25	2.53	5.03
5	0.40	0.95	1.25	1.55	4.15	1.30	2.90	3.40	3.40	11.00
6	0.25	1.40	2.50	3.05	7.50	0.00	0.40	0.85	1.10	2.35
7	1.00	1.90	3.65	4.55	10.90	0.15	0.20	0.30	0.50	1.15
8	0.30	1.10	1.85	2.20	5.45	1.15	1.75	2.10	2.35	7.35
9	1.05	2.10	3.96	6.50	13.61	0.10	0.60	1.20	1.55	3.45
10	0.90	1.45	1.90	5.45	9.70	0.20	0.75	0.95	1.75	3.65
11	0.90	1.40	2.68	3.85	8.83	0.95	1.85	2.25	2.35	7.40
12	0.75	2.95	3.35	4.95	12.00	0.30	0.95	1.50	1.90	4.65
13	0.45	1.60	2.30	4.70	9.05	0.00	0.60	1.11	1.68	3.39
14	0.00	0.60	2.20	2.70	5.50	0.30	0.70	1.20	1.62	3.82
15	0.40	0.90	1.20	1.95	4.45	0.35	0.35	0.45	1.15	2.30
16	1.50	3.55	3.95	5.85	14.85	0.50	0.60	1.10	2.70	4.90
17	0.25	0.30	0.60	1.30	2.45	0.10	0.40	0.45	1.30	2.25
18	1.50	2.70	4.00	6.45	14.65	0.65	0.70	0.70	1.50	3.55
19	1.15	2.20	2.90	4.35	10.60	0.20	0.80	1.42	2.65	5.07
20	1.65	3.88	4.60	6.00	16.13	1.10	1.30	2.25	4.38	9.03
21	0.50	1.65	2.00	3.70	7.85	0.55	0.78	0.88	2.05	4.26
22	0.40	1.10	1.55	2.95	6.00	0.10	0.10	0.80	1.21	2.21
23	0.45	0.75	2.02	4.95	8.17	0.60	0.95	1.32	2.55	5.42
24	0.35	1.30	2.25	3.45	7.35	1.30	1.70	2.25	3.00	8.25
25	2.40	2.85	4.10	5.25	14.60	0.45	1.20	1.30	1.75	4.70
26	0.65	2.50	2.70	2.85	8.70	0.05	0.05	0.20	0.60	0.90
27	0.50	1.45	1.45	2.50	5.90	0.20	0.70	0.95	1.30	3.15
28	0.60	2.20	2.55	3.25	8.60	0.75	1.20	1.20	1.55	4.70
Period total.	21.35	48.38	69.76	104.10	243.59	13.35	24.15	35.41	55.67	128.58

From Table 2 the correction term (CT) is obtained as follows:

$$\text{CT} = \frac{(\text{Grand total of 224 readings})^2}{224} = \frac{(409.53)^2}{224} = 748.7269.$$

$$\text{Between individuals SS} = \frac{\text{Sum of 28 (ind. tot.)}^2}{(2)(4)} - \text{CT}$$

$$= \frac{(10.70)^2 + (12.45)^2 + \ldots + (8.25)^2 + (13.40)^2}{8} - \text{CT}$$

$$= 106.9602.$$

$$\text{Between periods SS} = \frac{\text{Sum of 4 (period tot.)}^2}{(2)(28)} - \text{CT}$$

$$= \frac{(41.48)^2 + (81.20)^2 + (117.59)^2 + (169.26)^2}{56} - \text{CT}$$

$$= 158.2443 .$$

$$\text{Between cells SS} = \frac{\text{Sum of 112 (cell reading)}^2}{2} - \text{CT}$$

$$= \frac{(0.90)^2 + (1.30)^2 + \ldots + (3.50)^2 + (5.10)^2}{2} - \text{CT}$$

$$= 228.4853 .$$

TABLE 2

PLASMA SALICYLATE LEVELS (MG. PER CENT) FOR DRUG B PLUS DRUG A, RUN 1

IND.	PERIODS (MINUTES)				IND. TOTAL
	10	15	20	30	
1.....	0.90	1.30	3.05	5.45	10.70
2.....	1.30	2.35	3.80	5.00	12.45
.	.	.	.	.	.
.	.	.	.	.	.
.	.	.	.	.	.
.	.	.	.	.	.
.	.	.	.	.	.
27.....	0.10	1.45	2.70	4.00	8.25
28.....	2.30	2.50	3.50	5.10	13.40
Period total..	41.48	81.20	117.59	169.26	409.53

Each individual-period cell reading represents the sum of a reading for drug B and a reading for drug A.

$$\text{Individual-period interaction SS} = \text{Bet. cells SS} - (\text{bet. ind. SS} + \text{bet. per. SS})$$

$$= 23.2808 .$$

From the individual totals specific for drug in Table 1*a*, we compute the following:

$$\text{Between drugs SS} = \frac{\text{Sum of 2 (drug tot.)}^2}{(28)(4)} - \text{CT}$$

$$= \frac{(274.00)^2 + (135.53)^2}{112} - \text{CT} = 85.5979$$

$$\text{Between cells SS} = \frac{\text{Sum of 56 (cell reading)}^2}{4} - \text{CT}$$

$$= \frac{(4.10)^2 + (6.60)^2 + \ldots + (9.00)^2 + (4.40)^2}{4} - \text{CT}$$

$$= 256.5801 .$$

Each individual-drug cell reading represents the sum of four period readings.

$$\text{Individual-drug interaction SS} = \text{Bet. cells SS} - (\text{bet. ind. SS} + \text{bet. drugs SS})$$
$$= 64.0220 .$$

From the period totals specific for drug in Table 1*a*, we obtain:

$$\text{Between cells SS} = \frac{\text{Sum of 8 (cell reading)}^2}{28} - \text{CT}$$
$$= \frac{(28.18)^2 + (13.30)^2 + \ldots + (110.94)^2 + (58.32)^2}{28} - \text{CT}$$
$$= 258.1900 .$$

Each period-drug cell reading represents the sum of 28 individual readings.

$$\text{Period-drug interaction SS} = \text{Bet. cells SS} - (\text{Bet. per. SS} + \text{bet. drugs SS})$$
$$= 14.3479 .$$

Thus far, we have obtained six of the requisite seven SS. The remaining term, individual-period-drug interaction SS, is obtained by subtracting the sum of the other six SS from the between-cells SS calculated from all the 224 cell readings, specific for individual, period, and drug:

$$\text{Between cells SS} = \text{Sum of 224 (cell reading)}^2 - \text{CT}$$
$$= (0.15)^2 + (0.25)^2 + \ldots + (1.40)^2 + (1.75)^2 - \text{CT}$$
$$= 471.8036$$

Each cell represents a single reading.

$$\text{Individual-period-drug interaction SS} = \text{Bet. cells SS} - \text{sum of six other SS}$$
$$= 471.8036 - 452.4531 = 19.3505 .$$

The SS of the three criteria (three-way) analysis of variance for the first run are given in Table 3, along with those which were calculated in similar manner, for the second run of the experiment. The four SS involving drugs can be checked very easily by an independent procedure, since there are only two drugs involved. For this purpose, a table of differences of the values of drug B minus drug A for each patient at each time period is constructed. Thus, for the first patient, the difference between B and A at 10 minutes is $0.15 - 0.75 = -0.60$; at 15 minutes, $0.25 - 1.05 = -0.80$; at 20 minutes, $1.35 - 1.70 = -0.35$; and at 30 minutes, $2.35 - 3.10 = -0.75$; and so on for the other 27 patients. A portion of this summary table appears as Table 4.

From this table, we calculate

$$\text{CT} = \frac{(\text{Grand total of 112 readings})^2}{(112)(2)} = \frac{(+138.47)^2}{224} = 85.5979 = \text{Bet. drugs SS} .$$

TABLE 3

SUMS OF SQUARES FOR THREE-WAY AND FOUR-WAY ANALYSIS OF VARIANCE

SOURCE OF VARIATION	DEGREE OF FREEDOM	SUMS OF SQUARES			MEAN SQUARE
		Run 1	Run 2	(Run 1 + Run 2) (Final)	
Between individuals (I)	(28−1)=27	106.9602	73.5401	128.9918	4.7775
Between periods (P)	(4−1)=3	158.2443	150.4329	308.5298	102.8433
Between drugs (D)	(2−1)=1	85.5979	59.0504	143.4199	143.4199
Individual-period interaction (IP)	(28−1)(4−1)=81	23.2808	24.9947	31.9546	0.3945
Individual-drug interaction (ID)	(28−1)(2−1)=27	64.0220	59.3815	77.2006	2.8593
Period-drug interaction (PD)	(4−1)(2−1)=3	14.3479	15.5296	29.7963	9.9321
Ind.-per.-drug interaction (IPD)	(28−1)(4−1)(2−1)=81	19.3505	19.5478	21.1327	0.2609
Individual-run interaction (IR)	(28−1)(2−1)=27	.....	.....	51.5085	1.9077
Period-run interaction (PR)	(4−1)(2−1)=3	.....	.....	0.1474	0.0491
Drug-run interaction (DR)	(2−1)(2−1)=1	.....	.....	1.2284	1.2284
Ind.-per.-run interaction (IPR)	(28−1)(4−1)(2−1)=81	.....	.....	16.3209	0.2015
Ind.-drug-run interaction (IDR)	(28−1)(2−1)(2−1)=27	.....	.....	46.2029	1.7112
Per.-drug-run interaction (PDR)	(4−1)(2−1)(2−1)=3	.....	.....	0.0812	0.0271
Ind.-per.-drug-run interaction (IPDR)	(28-1)(4-1)(2-1)(2-1)=81	.....	.....	17.7656	0.2193
Between runs (R)	(2-1)=1	.....	.....	3.1156	3.1156
Total	(28)(4)(2)(2)−1=447	.....	.....	877.3962	.....

TABLE 4

DIFFERENCE IN PLASMA SALICYLATE LEVELS (MG. PER CENT)
DRUG B−DRUG A, RUN 1

IND.	PERIODS (MINUTES)				IND. TOTAL
	10	15	20	30	
1	− 0.60	− 0.80	− 0.35	− 0.75	− 2.50
2	+ 1.10	+ 2.05	+ 2.80	+ 3.50	+ 9.45
.	.	.	.	.	.
.	.	.	.	.	.
.	.	.	.	.	.
.	.	.	.	.	.
.	.	.	.	.	.
27	− 0.10	− 0.55	+ 0.30	+ 1.40	+ 1.05
28	+ 1.10	+ 1.20	+ 0.70	+ 1.60	+ 4.60
Period total	+14.88	+28.84	+42.13	+52.62	+138.47

Between individuals SS

$$= \frac{\text{Sum of } 28 \text{ (ind. tot.)}^2}{(4)(2)} - \text{CT}$$

$$= \frac{(-2.50)^2 + (+9.45)^2 + \ldots + (+1.05)^2 + (+4.60)^2}{8} - \text{CT}$$

$$= 64.0219 = \text{Individual-drug interaction SS}.$$

$$\text{Between periods SS} = \frac{\text{Sum of } 4 \text{ (per. tot.)}^2}{(28)(2)} - \text{CT}$$

$$= \frac{(+14.88)^2 + (+28.84)^2 + (+42.13)^2 + (+52.62)^2}{56} - \text{CT}$$

$$= 14.3478 = \text{Period-drug interaction SS}.$$

$$\text{Between cells SS} = \frac{\text{Sum of } 112 \text{ (cell reading)}^2}{2} - \text{CT}$$

$$= \frac{(-0.60)^2 + (-0.80)^2 + \ldots + (+0.70)^2 + (+1.60)^2}{2} - \text{CT}$$

$$= 97.7203.$$

Each individual-period cell involves a difference between two drugs and, hence, two readings.

$$\text{Individual-period interaction SS} = \text{Bet. cells SS} - (\text{bet. per. SS} + \text{bet. ind. SS})$$

$$= 19.3506 = \text{Ind.-per.-drug interaction SS}.$$

Four-way analysis of variance on both runs.—In order to calculate the SS for the fifteen sources of variation in the four-way analysis of variance, we find it convenient to compute, first, the seven SS corresponding to a three-way analysis on the first run combined with the second run. For this purpose, we need to construct two additional

TABLE 5

SUM OF PLASMA SALICYLATE LEVELS (MG. PER CENT) OF DRUG B FOR BOTH RUNS AND DRUG A FOR BOTH RUNS

IND.	DRUG B					DRUG A				
	10 min.	15 min.	20 min.	30 min.	Ind. Total	10 min.	15 min.	20 min.	30 min.	Ind. Total
1	0.80	2.20	3.65	6.75	13.40	1.75	2.17	3.70	6.70	14.32
2	1.60	2.65	3.90	4.95	13.10	0.10	0.30	1.40	2.15	3.95
.	.	.	.	.	.	.	.	.	.	.
.	.	.	.	.	.	.	.	.	.	.
.	.	.	.	.	.	.	.	.	.	.
.	.	.	.	.	.	.	.	.	.	.
27	0.50	1.90	2.95	5.20	10.55	0.30	1.70	2.15	2.60	6.75
28	2.30	4.05	4.65	6.60	17.60	1.35	1.85	2.60	3.30	9.10
Period total.	49.53	103.40	149.62	215.04	517.59	26.65	50.33	73.14	113.99	264.11

tables in which corresponding readings for the two runs are added together. A table is constructed in which the corresponding readings of Table 1*a* are added to those of Table 1*b*. Thus, for the first individual with drug B, at 10 minutes, $0.15 + 0.65 = 0.80$; at 15 minutes, $0.25 + 1.95 = 2.20$; etc. For the first individual with drug A, at 10 minutes, $0.75 + 1.00 = 1.75$; at 15 minutes, $1.05 + 1.12 = 2.17$; etc. A portion of this summary table appears as Table 5. Another summary table similar to Table 2 is constructed in which, for each individual at each time period, we have the sum of four readings (drug B first run plus drug A first run plus drug B second run plus drug A second run). A portion of this table appears as Table 6.

From Table 6, we calculate

$$\text{CT} = \frac{(\text{Grand total of 56 cell readings})^2}{448} = \frac{(781.70)^2}{448} = 1363.9618.$$

$$\text{Between individuals SS} = \frac{\text{Sum of 28 (ind. tot.)}^2}{(4)(4)} - \text{CT}$$

$$= \frac{(27.72)^2 + (17.05)^2 + \ldots + (17.30)^2 + (26.70)^2}{16} - \text{CT}$$

$$= 128.9918.$$

$$\text{Between periods SS} = \frac{\text{Sum of 4 (per. tot.)}^2}{(4)(28)} - \text{CT}$$

$$= \frac{(76.18)^2 + (153.73)^2 + (222.76)^2 + (329.03)^2}{116} - \text{CT}$$

$$= 308.5298.$$

$$\text{Between cells SS} = \frac{\text{Sum of 112 (cell reading)}^2}{4} - \text{CT}$$

$$= \frac{(2.55)^2 + (4.37)^2 + \ldots + (7.25)^2 + (9.90)^2}{4} - \text{CT}$$

$$= 469.4762.$$

TABLE 6

SUM OF PLASMA SALICYLATE LEVELS (MG. PER CENT) OF (DRUG B+ DRUG A, RUN 1) AND (DRUG B+DRUG A, RUN 2)

IND.	PERIODS (MINUTES)				IND. TOTAL
	10	15	20	30	
1.....	2.55	4.37	7.35	13.45	27.72
2.....	1.70	2.95	5.30	7.10	17.05
.	.	.	.	.	.
.	.	.	.	.	.
.	.	.	.	.	.
.	.	.	.	.	.
.	.	.	.	.	.
27.....	0.80	3.60	5.10	7.80	17.30
28.....	3.65	5.90	7.25	9.90	26.70
Period total.	76.18	153.73	222.76	329.03	781.70

Each individual-period cell reading represents the sum of four readings.

$$\text{Individual-period interaction SS} = \text{Bet. cells SS} - (\text{bet. ind. SS} + \text{bet. per. SS})$$
$$= 31.9546 .$$

From the individual totals specific for drug in Table 5 we calculate

$$\text{Between drugs SS} = \frac{\text{Sum of 2 (drug tot.)}^2}{(2)(112)} - \text{CT}$$
$$= \frac{(517.59)^2 + (264.11)^2}{224} - \text{CT} = 143.4199 .$$

$$\text{Between cells SS} = \frac{\text{Sum of 56 (cell reading)}^2}{8} - \text{CT}$$
$$= \frac{(13.40)^2 + (14.32)^2 + \ldots + (17.60)^2 + (9.10)^2}{8} - \text{CT}$$
$$= 349.6123 .$$

Each individual-drug cell reading represents the sum of eight readings.

$$\text{Individual-drug interaction SS} = \text{Bet. cells SS} - (\text{bet. ind. SS} + \text{bet. drugs SS})$$
$$= 77.2006 .$$

From the period totals specific for drug in Table 5, we calculate

$$\text{Between cells SS} = \frac{\text{Sum of 8 (cell total)}^2}{56} - \text{CT}$$
$$= \frac{(49.53)^2 + (103.40)^2 + \ldots + (73.14)^2 + (113.99)^2}{56} - \text{CT}$$
$$= 481.7460 .$$

Each period-drug cell reading represents the sum of fifty-six readings.

$$\text{Period-drug interaction SS} = \text{Bet. cells SS} - (\text{bet. drugs SS} + \text{bet. per. SS})$$
$$= 29.7963 .$$

Thus far, we have obtained six of the requisite seven SS for the three-way analysis of variance of the first run combined with the second run. The remaining term, individual-period-drug interaction SS, is obtained by subtracting the sum of the other six SS from the between cells SS calculated from 224 cell readings specific for individual, period, and drug. From Table 5, we calculate

$$\text{Between cells SS} = \frac{\text{Sum of 224 (cell reading)}^2}{2} - \text{CT}$$
$$= \frac{(0.80)^2 + (2.20)^2 + \ldots + (2.60)^2 + (3.30)^2}{2} - \text{CT}$$
$$= 741.0257 .$$

Each cell represents the sum of two readings, i.e., first run plus second run.

Individual-period-drug interaction SS = Bet. cells SS − sum of six other SS

$$= 741.0257 - 719.8930 = 21.1327 .$$

These seven SS appear in the next to the last column of Table 3.

There are fifteen terms in the four-way analysis of variance, of which seven have already been calculated. Of the remaining eight terms, seven refer to the interaction of the seven terms already obtained, with run. The other one refers to the difference between runs. These eight additional terms are also given in Table 3. It will be noted that the degrees of freedom for all the interaction terms involving run are exactly the same as for the original seven terms in this table because there are only two runs and hence there are $(2 - 1)$ degrees of freedom for between runs SS.

The seven interaction terms involving run are readily obtained by adding together the corresponding SS of run 1 and of run 2 (Table 3) and subtracting the SS for (run 1+ run 2). Thus (between individuals SS, run 1 + between individuals SS, Run 2) −(between individuals SS, Run 1 + Run 2) = Individual—run interaction SS = (106.9602 + 73.5401) −128.9918 = 51.5085. Similarly, (individual-drug interaction SS, run 1 + individual-drug interaction SS, run 2) − (individual-drug interaction SS, run 1 + run 2) = Individual-drug-run interaction SS = (64.0220 + 59.3815) − 123.4035 = 46.2029, and so on for the other five terms.

The fifteenth and last term in the four-way analysis of variance is between runs SS and is obtained as the sum of the CT for run 1 and the CT for run 2 minus the CT for (run 1 + run 2). Thus

$$\text{Between-runs SS} = \frac{(409.53)^2}{224} + \frac{(372.17)^2}{224} - \frac{(781.70)^2}{448} = 3.1156 .$$

Various independent checks of some of the values of the SS may be made. For example, since there are only two runs and two drugs, the four terms in Table 3 involving drug-run may easily be checked by using a table of differences of differences. A table is constructed in which, for each individual at each time period, the difference between the reading for drug B minus drug A for the second run is subtracted from the corresponding difference between the reading for drug B minus drug A for the first run. Thus, for the first individual at 10 minutes, $(0.15 - 0.75) - (0.65 - 1.00) = -0.25$; at 15 minutes, $(0.25 - 1.05) - (1.95 - 1.12) = -1.63$; at 20 minutes, $(1.35 - 1.70) - (2.30 - 2.00) = -0.65$; and at 30 minutes, $(2.35 - 3.10) - (4.40 - 3.60) = -1.55$; and so on for the other 27 individuals. A portion of this table appears as Table 7. From this table, we calculate

$$\text{CT} = \frac{(\text{Grand total of 112 cell reading})^2}{(112)(4)} = 1.2285 = \text{drug-run interaction SS}$$

Between individuals SS

$$= \frac{\text{Sum of 28 (ind. tot.)}^2}{(4)(4)} - \text{CT}$$

$$= \frac{(-4.08)^2 + (+9.75)^2 + \ldots + (-1.70)^2 + (+0.70)^2}{16} - \text{CT}$$

$$= 46.2027 = \text{Ind.-drug-run interaction SS} .$$

$$\text{Between periods SS} = \frac{\text{Sum of 4 (per. tot.)}^2}{(28)(4)} - \text{CT}$$

$$= \frac{(+6.88)^2 + (+4.61)^2 + (+7.78)^2 + (+4.19)^2}{112} - \text{CT}$$

$$= 0.0811 = \text{Per.-drug-run interaction SS}.$$

$$\text{Between cells SS} = \frac{\text{Sum of 112 (cell reading)}^2}{4} - \text{CT}$$

$$= \frac{(-0.25)^2 + (-1.63)^2 + \ldots + (-0.65)^2 + (-0.10)^2}{4} - \text{CT}$$

$$= 64.0496.$$

Each individual-period cell represents a difference involving four original readings.

Individual-period SS

= Between cells SS − (between periods SS + between individuals SS)

= 17.7658 = Individual-period-drug-run interaction SS.

The values for the mean square (MS) in the last column of Table 3 are obtained by dividing the SS by the number of degrees of freedom for the particular source of variation.

INTERPRETATIONS

While the calculations of the fifteen components in the four-way analysis of variance can be performed rather readily, considerable difficulty may be encountered in the selection of the appropriate numerator and denominator for the *F*-ratios, which are used for testing the statistical significance of the various effects or interactions. The numerator is the particular effect or interaction that the investigator is interested in testing, with its degrees of freedom, d_1. The difficulty usually lies in the selection of the appropriate denominator, i.e., the error MS, with its degrees of freedom, d_2. For exam-

TABLE 7

DIFFERENCES IN PLASMA SALICYLATE LEVELS (MG. PER CENT)
(DRUG B − DRUG A, RUN 1) − (DRUG B − DRUG A, RUN 2)

IND.	PERIODS (MINUTES)				IND. TOTAL
	10	15	20	30	
1	−0.25	−1.63	−0.65	−1.55	− 4.08
2	+0.70	+1.75	+3.10	+4.20	+ 9.75
.	.	.	.	.	.
.	.	.	.	.	.
.	.	.	.	.	.
.	.	.	.	.	.
27	−0.40	−1.30	−0.20	+0.20	− 1.70
28	+1.25	+0.20	−0.65	−0.10	+ 0.70
Period total.	+6.88	+4.61	+7.78	+4.19	+23.46

ple, do the two drugs differ with respect to the level of plasma salicylate obtained? In this case the numerator for the F-ratio is obviously between drugs MS, $d_1 = 1$. In this experiment, where the only random criterion is individual, the appropriate denominator for testing the difference between drugs is that term which measures how the drug difference fluctuates from individual to individual, namely, individual-drug MS (ID), $d_2 = 27$.

The selection of the appropriate error term, i.e., the denominator of the F-ratio, will depend upon the type of model to which the experiment conforms. In our example, on which the four-way analysis of variance is based, there are four criteria: drugs (D), periods (P), individuals (I), and runs (R). The investigators were interested in two specific drugs and the plasma levels of salicylate at four specific time periods. They selected 28 individuals at random from some universe of all individuals who could have been selected, and they performed two specific runs of the experiment. Thus we shall consider the criteria drug, period, and runs as "fixed" and the criterion individuals as "random." This is a simple extension of the model for two fixed and one random criteria.[4]

In this type of model, with three criteria fixed and one random, the error MS (the denominator of the F-ratio) for any effect or interaction is the interaction of that same effect (or interaction) with individual (I), the random criterion. There are simple rules of thumb for determining the expected values of the mean squares and hence for selecting the appropriate numerators and denominators for the F-tests under the various models.[5] If the effect (or interaction) includes I, then there is no proper denominator unless certain higher-order interactions can be assumed to be truly zero. However, we were not usually interested in testing the sources of variation involving I because it was recognized at the outset that there would be individual differences. It was precisely for this reason that this "nuisance" variable, I, was controlled in the design by cross-classifying the other criteria with individual, i.e., by using the same individuals over again.

In the plasma salicylate experiment used as an example, the investigators were primarily interested in answering three questions:

1. Does the plasma salicylate level change with time, regardless of drug? In other words, is the absorption curve depicted in Figure 1 ($B + A$ curve) different from a horizontal line?

2. Is there a difference between the two drugs in the plasma salicylate level obtained, regardless of time? This question calls for a comparison of the average readings over the four time periods for drug B with those of drug A (Fig. 1).

3. Is the change in plasma salicylate level with time different for the two drugs? This question refers to the parallelism of the two absorption curves as well as to the constancy of the drug differences for the various periods (Fig. 1).

In answering the first question, the numerator of the F-ratio is obviously between periods MS (P). The denominator is the interaction of period with individual, i.e., individual-period interaction MS (IP). Thus

$$F_1 = \frac{\text{P}}{\text{IP}} = \frac{102.8433}{0.3945} = 260.69; \qquad d_1 = 1, \qquad d_2 = 81 \text{ (significant)}.*$$

* The values of F exceeded 1 and 5 per cent of the time by chance for various combinations of degrees of freedom appearing in standard tables of the *F-distribution* (see ref. 6).

The absorption curve $(B + A)$ in Figure 1 differs from a horizontal line.

Similarly, the numerator of the F-ratio for answering question 2 is between drugs MS (D), and the denominator is the interaction of drug with individual, i.e., individual-drug interaction MS (ID):

$$F_2 = \frac{\text{D}}{\text{ID}} = \frac{143.4199}{2.8593} = 50.16; \qquad d_1 = 1, \qquad d_2 = 27 \quad \text{(significant)}.$$

The B and the A curves in Figure 1 are at different vertical levels.

The third question is answered by

$$F_3 = \frac{\text{PD}}{\text{IPD}} = \frac{9.9321}{0.2609} = 38.17; \qquad d_1 = 3, \qquad d_2 = 81 \quad \text{(significant)}.$$

The B and the A curves in Figure 1 are not parallel.

Actually, questions 2 and 3 are more important for the investigators than question 1, since they know from prior experience that there is some sort of plasma salicylate absorption curve. In general, there are four possible combinations of significance and nonsignificance of the ratios referring to questions 2 and 3:

A. D/ID significant and PD/IPD significant.—There is an average drug difference over the time periods, but the average difference is not constant, i.e., the absorption

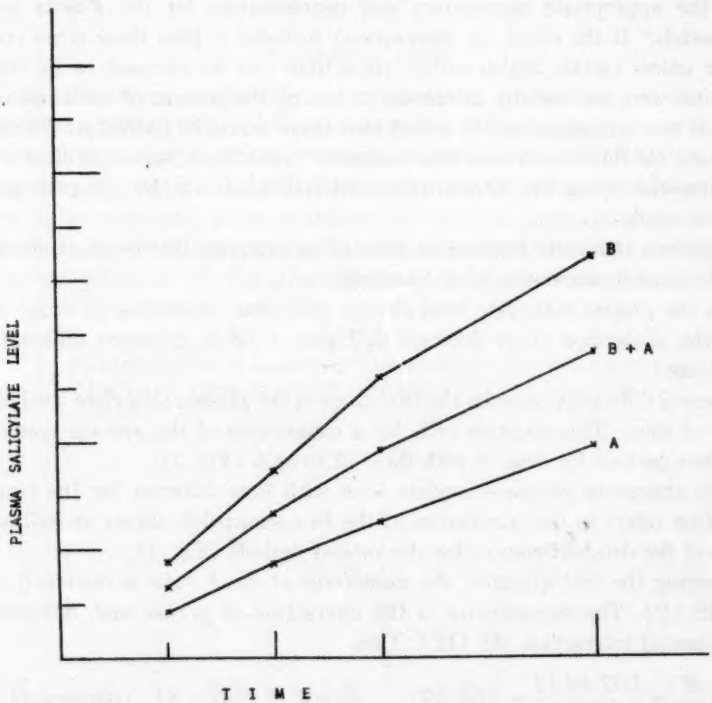

FIG. 1.—D/ID significant; PD/IPD significant

curves are not parallel. This is the actual situation in the plasma salicylate example, as shown in Figure 1. Drug B gives higher plasma salicylate levels and has a faster absorption curve than Drug A.

B. D/ID significant and PD/IPD not significant.—There is a drug difference which is constant, within chance error, from period to period, i.e., the absorption curves are parallel, without consideration of their shapes (see Fig. 2 for hypothetical examples).

C. D/ID not significant and PD/IPD significant.—The two absorption curves are different, but there is some sort of compensation (or crossing over), so that, over the periods studied, there is no significant difference between the average drug readings (see Fig. 3 for hypothetical examples).

D. D/ID not significant and PD/IPD not significant.—There is no average drug difference for the periods, and the curves are parallel, i.e., the curves essentially coincide except for chance variation (see Fig. 4 for hypothtical examples).

Before we can accept the above exposition of drug differences, it is necessary to investigate the consistency of the findings for the two runs. For this purpose various significance tests of the MS related to the sources of variation involving runs may be made. The various questions may be listed as follows:

1. Is the plasma salicylate level the same for the two runs?
2. Is the shape of the absorption curve the same for the two runs?
3. Is the difference between the two drugs the same for the two runs?

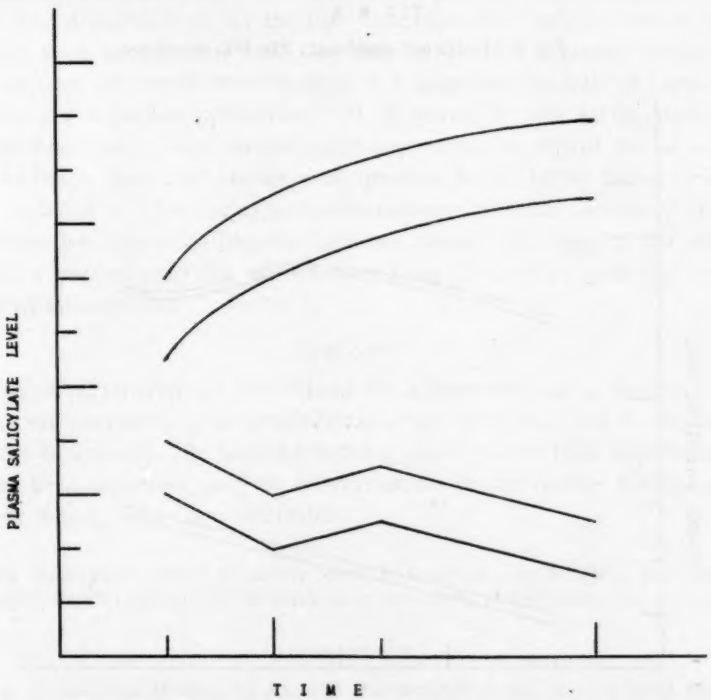

FIG. 2.—D/ID significant; PD/IPD not significant

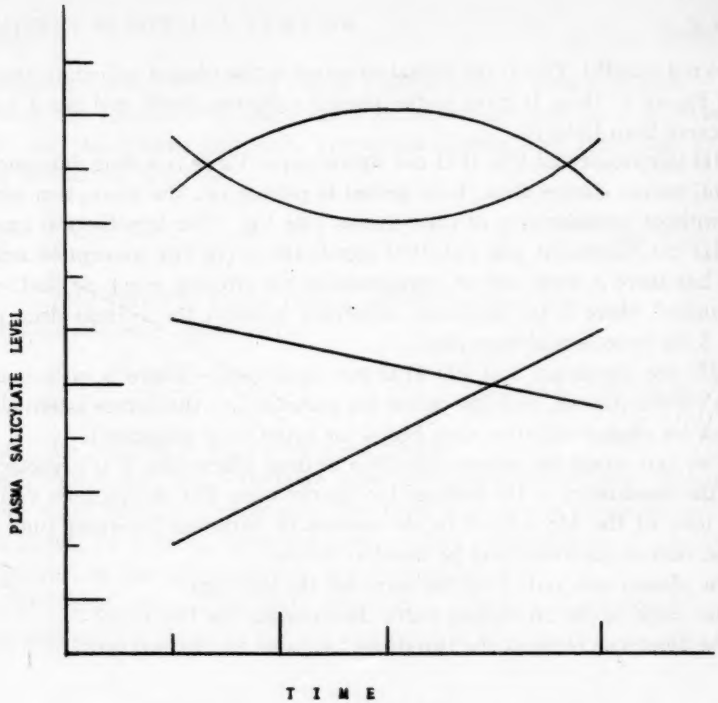

FIG. 3.—D/ID not significant; PD/IPD significant

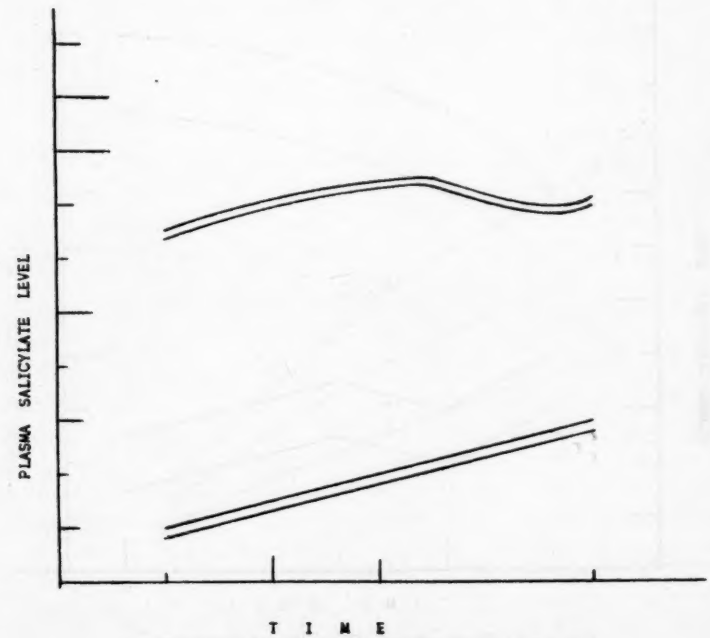

FIG. 4.—D/ID not significant; P/IPD not significant

4. Is the drug difference in the shape of the absorption curve the same for the two runs?

To answer these questions, it is necessary to form *F*-ratios in which the numerator is the appropriate effect (or interaction) involving runs, and the denominator is the interaction of that same effect (or interaction3 with individual:

$$F_1 = \frac{\text{R}}{\text{IR}} = \frac{3.1155}{1.9077} = 1.63; \qquad d_1 = 1, \qquad d_2 = 27 \quad \text{(not significant)};$$

$$F_2 = \frac{\text{PR}}{\text{IPR}} = \frac{0.0492}{0.2015} = 0.24; \qquad d_1 = 3, \qquad d_2 = 81 \quad \text{(not significant)},$$

$$F_3 = \frac{\text{DR}}{\text{IDR}} = \frac{1.2285}{1.7112} = 0.72; \qquad d_1 = 1, \qquad d_2 = 27 \quad \text{(not significant)};$$

$$F_4 = \frac{\text{DPR}}{\text{IDPR}} = \frac{0.0271}{0.2193} = 0.12; \qquad d_1 = 3, \qquad d_2 = 81 \quad \text{(not significant)}.$$

The fact that none of these F-ratios is significant allows us to accept the answers already given to the three original questions. If any of these latter *F*-ratios had been significant, it would have meant that some feature of the experiment was not repeatable from one run to another, thereby detracting from the validity of the experiment. Since four significance tests involving run have been made, there is some merit in making them at a more stringent level, say the 1 per cent instead of the 5 per cent level.

Sometimes when there are two runs, they may be regarded as merely random repetitions. In our case this would have resulted in a three-way analysis of variance with two readings per individual-period-drug cell. However, in view of the time interval between the two runs, it was deemed more appropriate to regard run as a definite criterion. At times, there may also be some question as to whether this criterion, run, should be regarded as a fixed or as a random criterion. We chose to regard it as fixed because these two runs constitute the first and second experience of the individual rather than a random selection of two runs from all possible runs that might be performed on an individual.

SUMMARY

A method of performing the calculations for a three-way and a four-way analysis of variance on completely cross-classified data has been described in the case of a mixed-model experiment. The basis for forming the *F*-ratios of the appropriate mean squares has been explained, and the interpretations of the various *F*-tests and combinations of *F*-tests have been elucidated.

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Mesial Margin Ridging of Incisor Labial Surfaces

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Specific morphological characters of the dentition which are believed to have a genetic basis are of particular importance in such areas of research as human evolution, population comparisons, and racial investigations. Such traits offer potential assistance for genetic differentiation of both living and archeological populations. The presence or lack of the structural formations commonly referred to as "shovel-shaped", manifested by ridges of the lateral borders on the lingual surface of incisor teeth, have often been cited as one such criterion for the determination of racial affinity in man.

Hrdlicka,[1] in a paper which brought together most previous knowledge of this feature, termed marked lateral borders of the lingual surface of incisors as resembling "more or less an ordinary coal shovel, in consequence of which they were termed 'shovel-shaped' incisors." A high incidence of this trait has come to be associated with individuals of Mongoloid affinity. American Indians have generally been reported to have an extremely high percentage of occurrence of the shovel-shaped form ranging from about 87 to 100 per cent (Hrdlicka,[1] Dahlberg,[2, 3] Hooton,[4] Lasker,[5] Nelsen,[6] Rabkin,[7] Wissler[8]), although a few exceptions have been found, such as that of Upper Gila Indians, for whom Nesbitt[9] reported an incidence of only 25.0 per cent in the females. White and Negroes are usually considered to have a much lower incidence of this character than Mongoloid populations.

However, the incidence and range of variation of this trait in some Caucasoid populations may be found to be higher than has been previously suspected in view of the findings of Lasker[4] and particularly the study of Koski and Hautala,[10] in which Finns were found to have a 91 per cent incidence. Most general statements citing the lowness of frequency in whites appear to be based upon the 1920 data of Hrdlicka of 52.4 per cent in white males and 44.6 per cent in white females, since, except for the above studies, there has been no further study of this trait in white populations.

In 1947 Dahlberg[2] described the "double shovel-shape" in which these marked lateral borders of incisor teeth were found on the labial, as well as on the lingual, surfaces of Pima Indians.

Recently still another morphological character involving the marginal areas of labial surfaces has been found on human incisor teeth during an investigation of the dental morphology of a prehistoric Indian population at the University of Arizona.[11]

MATERIAL AND OBSERVATIONS

The material consisted of a skeletal series of some 350 individuals, representing a time depth of about 350 years, from A.D. 1100 to A.D. 1450. These Point of Pines In-

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dians occupied a portion of what is now the San Carlos Apache Indian Reservation of east-central Arizona. Radiocarbon dating techniques of cremation finds at the nearby Cienega site[12] indicate that this area had been occupied as early as 2375 B.C.; however, it was not until the burials of A.D. 1100 that teeth useful for study were found.

During the course of this study it was found that a number of the incisor teeth did not fit the current classifications as to structural features. Upon closer investigation it was observed that this was due to a unique structural formation believed to be previously undescribed in the literature. This feature could perhaps most descriptively be termed the "three-quarter double shovel-shape" character if the nomenclature of Hrdlicka and Dahlberg were to be followed. However, since this is rather a ponderous term, a simple designation of such structural formation on incisor teeth as "mesial-ridged labial," or "MRL," may be more useful.

"Mesial-ridged labial" refers to the presence of a ridge on the mesial margin of the labial surface of incisor teeth running gingivally from the incisal edge. Teeth bearing

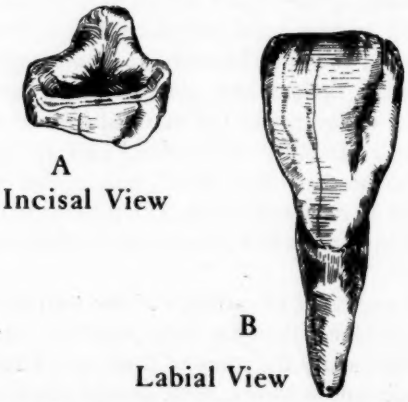

FIG. 1.—Illustration of the mesial-ridged labial character

this feature have also been observed to have a typical shovel-shape on the concave lingual surface. This ridge or elevation is similar to that characterizing a typical double shovel-shaped form except that the distal labial border is rounded rather than being elevated and there is no associated depression on the distal labial surface. Thus one half of the labial surface could be described as adhering to the double shovel-shaped form, while the distal portion appears normal, i.e., has no ridge. The single labial ridge gives the visual impression that the incisal edge is out of line with the body of the tooth (Figs. 1 and 2).

Figure 3 shows incisal views of five shovel-shaped incisor teeth. The one on the left has no labial ridging; the second, third, and fourth teeth show the MRL structure, which appears only on the mesial of the surface; and the fifth tooth is the double shovel-shaped form with the lateral ridges on both borders of the labial surface. The dentin exposed, seen in the third tooth, shows that such MRL ridging is not simply an enamel extension but is an integral part of the tooth structure.

This MRL feature was observed to be present on 24 incisor teeth of 13 individual

Point of Pines Indians, which represents an incidence of 6.5 per cent of the 200 individuals having either maxillary or mandibular incisor teeth present. Five of these individuals were earlier inhabitants of the time period from A.D. 1100 to A.D. 1250, and 8 were later Point of Pines Indians of A.D. 1250–1450. Seventeen of the 24 teeth showing this character were upper central incisors. However, it was also found on mandibular central and lateral incisors, although never so marked.

On each of these 24 teeth from the Point of Pines Indians, plus a subsequent case found in a mixture of contemporary extractions (which may be from either a Papago or San Carlos Apache individual), the ridging was observed to appear on the mesial-labial border only.

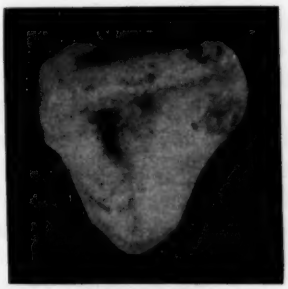

FIG. 2.—Incisal view of the holotype of this character, showing bilateral mesial-labial ridging on the maxillary central incisor of a twenty-two (±2)-year-old prehistoric Indian male from Point of Pines, Arizona (No. 115).

FIG. 3.—Incisal views of (*from left to right*) a "shovel-shaped" incisor having lingual marginal ridging only, three incisors showing MRL, and one example of a "double shovel-shaped" incisor. All the Point of Pines Indians showed the "shovel-shaped" feature on all incisors; 22.5 per cent also had the "double shovel-shaped" structure; and 6.5 per cent had the "mesial-ridged labial" formation.

Dahlberg[13] more recently has found one individual among the skeletal materials at the Chicago Natural History Museum that has, instead of the mesial-labial ridging, a distinct distal-labial ridging of the maxillary left central incisor. Thus it appears that this structure may represent a character midway between a double shovel-shaped form and a "normal" non-ridged labial incisor form, with a possibility of expression on either the mesial or the distal labial border.

It will be of interest in subsequent investigations to determine whether this feature is also found in other Mongoloid peoples and particularly what the incidence may be in various American Indian populations. No other mention of such an incisor structure in any population has been noted in the literature, except for brief mention by Moorrees[14] that "the labial shoveling frequently showed a greater accentuation of one of the two marginal ridges" in Aleuts. This also tends to indicate that not only the mesial but

rather either the mesial or the distal-labial border may contain a ridged structure in some populations.

Evidence already indicates that this feature will be found in other populations as well. In support of this, the excellent photographs of Indian Knoll maxillary incisors by Rabkin[10] show typical mesial-labial ridging of incisor teeth and also support the present finding that the raised marginal elevation appears on the mesial-labial aspect. In addition, two of the "ridged teeth" pictured by Hrdlicka[1] also appear to show this specific structural formation.

SUMMARY

A previously undescribed morphological character is reported upon which has been observed to be present on the mesial-labial aspect of 24 incisor teeth of 13 prehistoric Point of Pines Indians from east-central Arizona. This represents an incidence of 6.5 per cent of the 200 individuals in the sample of 350 from this population from which incisor teeth were recovered. Although only the phenotypic aspects of this character have been discussed, mesial margin ridging of incisor teeth may offer one additional trait useful for genetic differentiation of populations. Future investigations of the incidence of this trait in various populations, coupled with pedigree studies, may aid in clarification of the mode of inheritance of this dental structure.

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The Oral Yeast-Lactobacillus *Relationship*
III. *Growth Enhancement of* Lactobacillus casei *by* Candida albicans *and Saliva*

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One of the important factors contributing to the complex interrelationships between the components of the oral flora is the capacity of some members of this microbial community to provide certain nutrients that are essential to, and can be utilized by, other constituents of the population. The ability of *Candida albicans* to satisfy certain vitamin requirements of lactobacilli and other microörganisms in mixed cultures is an example of this type of syntrophic relationship.[1-3] Examination of filtrates from young yeast cultures has indicated that vitamins are released from actively growing yeast cells and that the resulting activity of the filtrates is not dependent upon the lysis of aging cells. In addition, by culturing the *Lactobacillus* in mixed culture with the yeast in an appropriately deficient medium, it can be shown that the growth of the bacterium is proportional to the concentration of the vitamins released by the yeast. It has also been observed that, with the exception of lysine, there is no release of amino acids into the surrounding menstrum by *Candida* in the same period of time and under the same conditions that permitted the accumulation of a maximum extracellular concentration of vitamins.[2]

Saliva, in general, has been shown to contain all the vitamins and many of the amino acids required by a number of oral microörganisms, as well as other factors which may exert inhibitory or stimulatory influences upon their growth.[2, 4, 5, 6-9] Some of these nutrients are, however, apparently present in saliva at limiting concentrations, as evidenced by the observation that when a vitamin-free medium is fortified with autoclaved saliva, the resultant growth of the microörganism is often limited. It has been shown that various amino acids that contribute to the nutrition of oral bacteria are sometimes present at suboptimal concentrations,[2] a characteristic that varies from sample to sample of saliva.

Since some of these observations were made under conditions that limited the period that the medium was exposed to *Candida*, in order to restrict the materials measured to those released by actively growing yeast cells additional experiments were carried

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out to determine the effect of a more prolonged conditioning of the medium. While the supply of vitamins and amino acids is of significance in the regulation of the population densities of certain members of the oral Protista, it would seem likely that such relatively complex entities as saliva and the growth products of a *Candida* would exhibit even broader influence upon the growth of oral microörganisms than could be described in terms of fortifying the growth medium with vitamins and amino acids per se. Such influences of yeast and saliva upon the growth characteristics of an oral *Lactobacillus* have been studied and are reported in the paper.

EXPERIMENTAL METHODS

Oral strains of *Lactobacillus casei* and *Candida albicans* were used. The inoculum of either microörganism was prepared by washing a 20–24-hour culture, grown in a partially defined (casamino acid) medium[2] containing 0.1 per cent glucose, and resuspending to the original volume in sterile distilled water. Each 10 ml. of test medium received 0.001 ml. of the resulting suspension of the appropriate microörganism.

The same partially defined, complete medium used to grow the inoculum, but with 2 per cent glucose, was conditioned by the growth of *C. albicans*. Preconditioning was accomplished in either a direct or a diphasic culture system. Direct conditioning of the medium was accomplished by growing the yeast in the medium and subsequently removing the cells by centrifugation and filtration through a sintered-glass filter. In the diphasic system described by Wilson and Goaz[2] the yeast was grown in a permeable viscose chamber suspended in the medium to be conditioned. Following appropriate conditioning periods, glucose concentrations were adjusted, and aliquots of the conditioned medium were inoculated with the *L. casei*. These cultures were incubated at 37° C., and growth of the *Lactobacillus* was measured turbidimetrically.

Mixed whole saliva was used throughout the study. It was collected by paraffin stimulation in large test tubes immersed in an ice bath, immediately pooled, autoclaved, and then added aseptically to the medium.

RESULTS AND DISCUSSION

In the diphasic culture system the ratio of the volume of medium in which the yeast are grown to that of the medium to be conditioned is limited and imposed a concentration factor that reduces the sensitivity of the system. Therefore, further to evaluate the validity of this culture system, a comparison was made of the amino acids released by the *Candida* growing within the viscose chamber of the diphasic system with those released by the *Candida* grown directly in the medium (Table 1). A chemically defined medium,[2] from which only those amino acids that are essential for the test *Lactobacillus* were omitted, was conditioned by *C. albicans* in both the direct and the diphasic system. No release of amino acids, except for lysine, was detectable after 3 days of conditioning in either system. However, when the conditioning period was extended to 6 days, it was found, as measured by single omission tests, that varying concentrations of the eleven essential amino acids had been released into the medium in both culture systems. Extension of the conditioning period to 9 days generally resulted in some additional increase in the concentration of each amino acid in the conditioned medium (Table 1). The detection of amino acids in the medium only after 6 days or longer exposure to the *Candida* suggests that they are not released in significant amounts by the

actively growing cultures of the yeast. It is probably the altered permeability of the aging cells that is responsible for the loss from the amino acid pool of the yeast.

In addition, it was observed that greater maximum growth of the *Lactobacillus* occurred in the complete medium conditioned directly by the yeast for either 6 or 9 days than in the same medium conditioned in the diphasic system. Also, in tests to determine the liberation of individual amino acids by the yeast, five of those found to be essential for the test *Lactobacillus*—cysteine, glutamic acid, lysine, phenylalanine, and tryptophane—were shown to be present at higher concentrations in the medium conditioned for 6 days by the direct method as compared to the diphasic system. By

TABLE 1

EFFECT OF PROLONGED INCUBATION AND MANNER OF CONDITIONING UPON RELEASE OF AMINO ACIDS INTO MEDIUM BY *C. Albicans*

Essential Amino Acid Omitted	Medium Conditioned for 6 Days				Medium Conditioned for 9 Days			
	Diphasic System		Direct System		Diphasic System		Direct System	
	Optical Density	Per Cent of Control	Optical Density	Per Cent of Control	Optical Density	Per Cent of Control	Optical Density	Per Cent of Control
1. Arginine	0.046	73	0.050	37	0.059	44	0.086	39
2. Cysteine	.040	63	.087	65	.124	93	.182	83
3. Glutamic acid	.020	32	.031	23	.037	28	.088	40
4. Isoleucine	.135	214	.139	104	.140	105	.183	84
5. Leucine	.118	187	.076	57	.163	122	.192	88
6. Lysine	.078	124	.097	72	.098	74	.115	53
7. Phenylalanine	.042	67	.065	48	.056	42	.066	30
8. Serine	.022	35	.015	11	.030	22	.050	23
9. Tryptophane	.030	48	.096	72	.124	93	.013	6*
10. Tyrosine	.067	106	.066	49	.120	90	.222	102
11. Valine	.081	128	.084	63	.100	75	.094	43
12. Control (none)	.063	100	.134	100	.133	100	.218	100
13. Control (all)	0.000	0	0.000	0	0.000	0	0.011	5

* This lower value at 9 days indicates a subsequent destruction or utilization of tryptophane.

contrast, however, when the two culture systems are compared on the basis of the percentage of growth that occurred in the appropriately conditioned control, from which no amino acids were omitted, the relative amino acid replacement obtained at 6 days in the diphasic culture system was substantially higher except in the case of cysteine and tryptophane (Table 1).

The greater growth-promoting activity of the medium conditioned directly by the yeast and sterilized by passage through a sintered-glass filter is probably more closely correlated to the greater mass of *Candida* cells that develop during the conditioning period than to any restricted diffusion of material through the viscose membrane; the inner viscose chamber of the diphasic culture system contains approximately 10 per cent of the total volume of assay medium in the complete system. This was substantiated, in part, by experiments in which it was determined that all the vitamins normally included in the culture medium and various sources of organic nitrogen, such as casamino acids, trypticase, and peptone, when placed within the viscose chamber,

rapidly diffuse and become equally distributed throughout the entire culture system.

When a nutritionally complete, partially defined (casamino acid) medium was conditioned in the diphasic system for 1, 2, and 3 days prior to inoculation with the *Lactobacillus,* little variation in maximum growth was observed (Fig. 1). However, extending the conditioning period to 5 days resulted in an additional increase in total growth of approximately 20 per cent. Although growth of the *Lactobacillus* in the control medium that had not been exposed to the *Candida* was still in the log phase at the time that the growth of the *Lactobacillus* in the yeast-conditioned medium had reached maximum, continued incubation resulted in a maximum turbidity equivalent to that observed in the 1-, 2-, and 3-day *Candida*-conditioned medium.

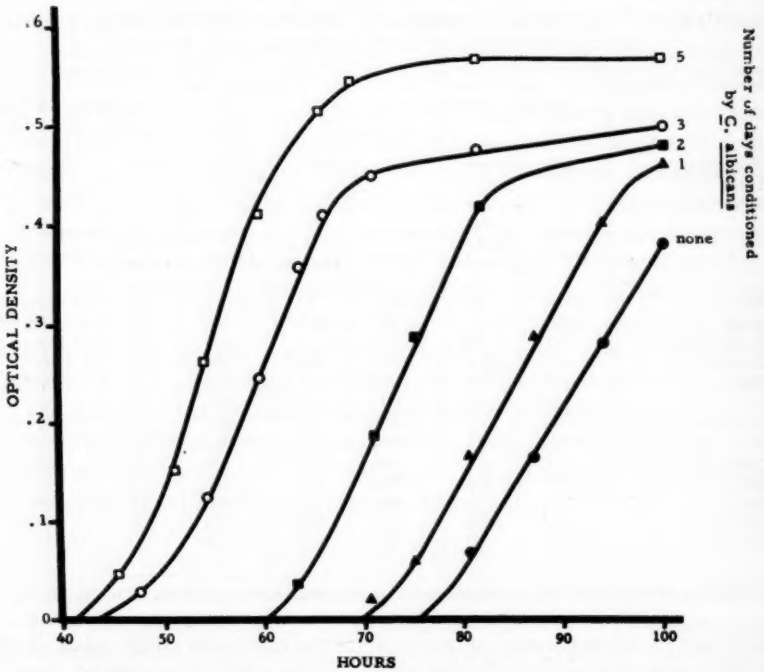

FIG. 1.—The influence of the period that a medium is conditioned by *C. albicans* upon the growth of *L. casei.*

The effect that conditioning the growth medium had on the initial stationary phase of the *Lactobacillus* was more striking than its effect on maximum growth (Fig. 1). A progressive reduction of lag time that was related to the period that the medium was exposed to the yeast was observed. This reduction was 8 per cent of the initial stationary phase observed in the control medium after conditioning for 1 day; a 20 per cent reduction after 2 days of conditioning; 40 per cent after 3 days; and 45 per cent after 5 days. A variation in the rate of growth was also observed in the yeast-conditioned medium. As the result of the same 1-, 2-, 3-, and 5-day conditioning periods the generation time was reduced 8.5, 29.5, 32, and 50 per cent, respectively.

The addition of saliva to the complete control medium permitted the development of

a greater maximum growth of the *Lactobacillus*, as well as shortening the initial stationary phase and increasing the rate of growth (Fig. 2). The addition of 10 per cent saliva to the medium decreased the initial stationary phase by approximately 20 per cent, while 25 per cent saliva reduced it by 30 per cent, and in both cases the generation time was decreased 35 per cent. In the absence of saliva, conditioning the medium with *Candida* for 3 days resulted in a decrease of 40 per cent in the initial stationary phase (Fig. 2). When saliva-supplemented medium was conditioned by the *Candida* for this same period (3 days), there were additional reductions, but these were some-

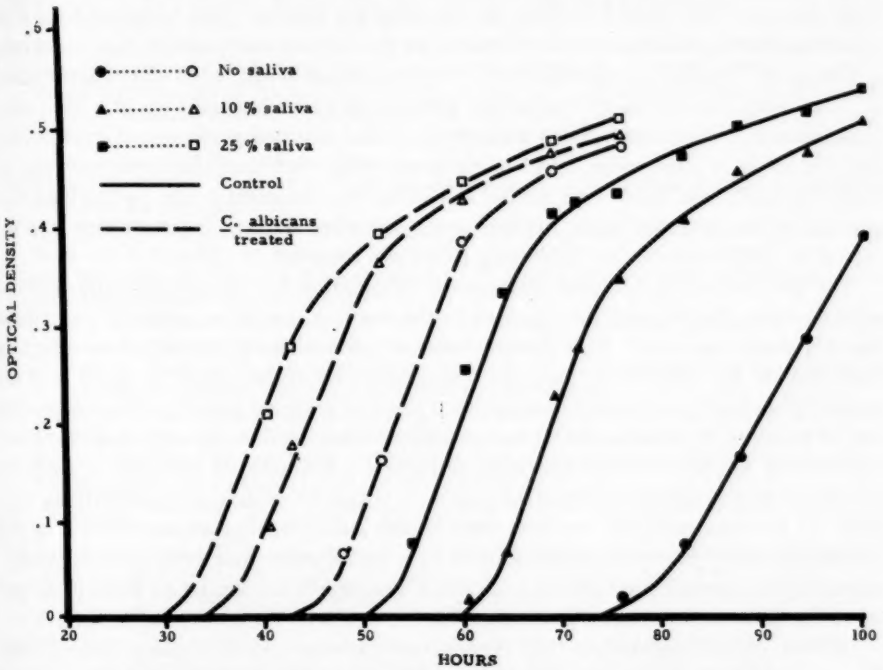

FIG. 2.—The combined effect of saliva-supplemented, *C. albicans*-conditioned medium upon the growth of *L. casei* (conditioning period 3 days).

what less than additive as compared with the two controls—52 per cent instead of 60 per cent in a 10 per cent saliva medium and 59 per cent instead of 70 per cent in the medium fortified with 25 per cent saliva. This less than additive response to the conditioned, saliva-supplemented medium is apparently a reflection of the utilization or destruction by the yeast of certain salivary constituents that are essential to the *Lactobacillus* or by the conditions attending the more prolonged preparatory periods. Such a reduction in the levels of salivary cysteine and phenylalanine-like activities by *Candida* has been observed in saliva-supplemented medium.[2] It was also demonstrated that the conditioning of both a 10 and a 25 per cent saliva-fortified medium resulted in a decrease in the initial stationary phase by 40 per cent as compared with the appropriate unconditioned, saliva-supplemented medium—the same value obtained by condition-

ing unsupplemented medium. This indicates that the effect that conditioning has upon the initial stationary phase is additive to the same effect of saliva.

In an appropriately deficient medium there is usually a close correlation between the amount of growth and the amount of acid produced by *Lactobacillus* cultures, both being, in turn, proportional to the concentration of essential nutrients.[10, 11] In a complete medium these relationships between growth and acid production and the availability of basic essential nutrients would not be demonstrable. That the concentration of the essential nutrients in the basal medium used in this study was at least optimal was indicated by the fact that increased concentrations of any one, or all, of the vitamins present in the growth medium did not alter the growth characteristics of the oral *Lactobacillus* in any manner. Furthermore, to the medium containing 0.5 per cent casamino acids, the addition of a mixture of nineteen amino acids,* each at a concentration of 100 μg/ml, as well as increasing the amounts of casamino acids up to a final concentration of 1.0 per cent, did not affect the initial stationary phase and growth rate but did result in a somewhat lower maximum growth when the higher concentration of casamino acids was used. This partial inhibition was apparently due to the high salt content of the casamino acids (14 per cent), for when the salt concentration was reduced by electrodialysis, the inhibitory effect was removed.

The mechanism of *Lactobacillus* growth stimulation by conditioning the medium with *Candida*, by the addition of saliva to the medium, and by a combination of both has not been elucidated. It is possible that an alteration of the oxidation-reduction potential of the medium is partially responsible. Extensive work by Snell and his group[13-17] has demonstrated the stimulatory effect of reducing agents and a preferential use of peptides by *lactobacilli*. It is not unlikely that similar materials are contained in saliva or are produced by the yeast in catalytic amounts. In fact, the addition of 25 μg/ml of an enzymatic digest of casein (trypticase), which contains various peptides, to the casamino acid medium used in this study resulted in a reduction in the initial stationary phase, increased growth rate, and greater maximum growth proportional to the concentration of this additive. These effects are similar to those produced by saliva and conditioning with the *Candida*.

In view of the observation that fermentable carbohydrate is usually cleared from the oral environment fairly rapidly,[18] it would seem that those factors which stimulate the activity of the acidogenic oral bacteria are probably of at least equal importance as those which exert their effect solely by permitting the development of a denser population. Of course, the optimum for maximum acid production would result from a combination of the effect of both these factors.

SUMMARY

A study of the capacity of an oral strain of *C. albicans* to fortify its growth medium with amino acids demonstrated that only lysine was detectable after 3 days' growth. However, after 6–9 days' incubation the medium had been supplemented with all the amino acids required by an oral strain of *L. casei*.

It was also noted that conditioning a medium with the *C. albicans* or supplementing

* L-cysteine, DL-serine, DL-isoleucine, L-glutamic acid, DL-tryptophane, DL-phenylalanine, L-leucine, DL-tyrosine, L-araginine, DL-valine, DL-alanine, L-asparagine, DL-aspartic acid, DL-methionine, glycine, L-histidine, DL-lysine, L-proline, L-hydroxyproline.

it with saliva appreciably decreased the initial stationary phase and increased the growth rate of the *Lactobacillus*. In addition, saliva was observed to increase maximum growth. This effect could be reproduced by addition to the medium of a digest of casein that had a relatively high concentration of peptides. It could not be duplicated by increasing vitamin and amino acid levels of the medium.

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Pyrophosphate: A Factor in Tooth Erosion

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Our interest in natural chelating agents in saliva and their possible effects on the erosion of tooth enamel led us to search for pyrophosphates. These substances have a wide distribution in both animal and plant tissues and are excellent chelating agents.

A previous report[1] showed that pyrophosphate solutions at pH from 5.0 to 7.5 are able to remove calcium from tooth enamel, leaving the surface smooth, as is found clinically in tooth erosion. It was speculated at that time that pyrophosphate might be produced in fermenting saliva. Such pyrophosphate accumulation, especially by salivary debris in contact with tooth substance, was suggested as a mechanism whereby eroded lesions on enamel, exposed dentin, and cementum might be produced.

Despite diligent investigation, we failed to find pyrophosphate in saliva or salivary debris, unless the material had first been allowed to incubate in the presence of low concentrations of NaF or hydroxylamine. This behavior suggested the presence of pyrophosphatase enzyme in the saliva or salivary debris because fluoride or hydroxylamine has been shown to inhibit that enzyme. The presence of pyrophosphatase, in the absence of suitable inhibitors, rapidly hydrolyzes any pyrophosphate that might be formed or accumulated in saliva. Therefore, no pyrophosphate would be detectable in the usual incubation mixture, or even in so-called fresh saliva samples.

We incubated salivary sediment or the microbial cells recovered when whole saliva aliquots were grown for 72 hours in either brain-heart infusion broth or thioglycolate medium, and we determined their pyrophosphatase activity by the method of Naganna and Menon[2] by incubating with pyrophosphate. In 1 hour, the debris or mixed microbial cells from 2 ml. of suspension were able to hydrolize 714 and 656 μg., respectively, of pyrophosphate. Boiled controls or reaction mixtures containing 0.001 M NaF did not split any of the pyrophosphate. The supernatant of centrifuged whole saliva was similarly pyrophosphatase-negative.

We now found that salivary debris as well as cultures of oral microbes can accumulate pyrophosphate while fermenting glucose, under favorable conditions. The amount thus accumulated is sufficient to remove calcium from tooth substance, even at nearly neutral pH.

This report presents data on two aspects of the problem: (*a*) accumulation of pyrophosphate by fermenting oral microbes and (*b*) decalcification of enamel, dentin, and cementum by pyrophosphate accumulated by fermentation mixtures.

EXPERIMENTAL METHODS AND RESULTS

a) Accumulation of pyrophosphate.—For this phase of the study, the accumulation of pyrophosphate by whole saliva, salivary debris, and mixed cultures of the microbial flora was investigated.

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1. Whole Saliva.—A mixture was made of 20 ml. pooled saliva, 2 ml. 0.4 M NaF, 2 ml. 0.1 M $MgCl_2$, and ml. 5 per cent glucose.

An aliquot was immediately treated with an equal volume of 10 per cent TCA and analyzed for orthophosphate by the method of Sumner.[3] This method determines orthophosphate but fails to react with pyrophosphate. The remainder was incubated for 24 hours at 38° C.

After incubating, an aliquot was again analyzed for orthophosphate as above. The remainder was treated with an equal volume of an aqueous solution of pyrophosphatase enzyme. This enzyme was prepared from erythrocytes according to the method of Naganna and Menon.[2] After 1 hour of incubating at 38° C., another aliquot was deproteinized and analyzed for orthophosphate. Of the orthophosphate that disappears during fermentation, that which reappears on treatment with pyrophosphatase enzyme is a measure of the pyrophosphate produced. Boiled saliva acted as a control in each step outlined above. The results of this part of the study are summarized in Table 1.

TABLE 1

PYROPHOSPHATE ACCUMULATION BY FERMENTING WHOLE SALIVA

	MG. PER 100 ML. (RANGE)	
	Boiled	Active
Initial ortho-P	100 (standard)	100 (standard)
Final ortho-P	100	81–93
Ortho-P regained by pyrophosphatase action	0	1.3–2.7
Pyrophosphate accumulated	0	7.4–15.4

2. Salivary Debris.—In a typical determination, 15 ml. of whole saliva were centrifuged for 5 minutes at 3000 rpm. The supernatant was decanted, the debris washed with 10 ml. isotonic saline solution, recentrifuged, and the supernatant discarded.

To the centrifugate in the tube, 1.5 ml. of 0.1 M Na_2HPO_4 in tris buffer, pH 7.6; 1.5 ml. of 0.1 M $MgCl_2$; 1.5 ml. of 0.4 M NaF; 1.5 ml. of 5 per cent glucose; and 6 ml. of tris buffer pH 7.6 were added and mixed.

Two milliliters of the reaction mixture were treated with 2.0 ml. of 10 per cent trichloroacetic acid, centrifuged, and the orthophosphate content determined. The remainder was incubated at 38° C. for 4 hours, when an aliquot was deproteinized and analyzed for orthophosphate.

The remainder was treated with an equal volume of 10 per cent TCA and homogenized in a Potter tissue homogenizer.

An aliquot was analyzed for orthophosphate and the remainder heated in a boiling water bath for 10 minutes. This treatment hydrolyzes the pyrophosphate to orthophosphate. The orthophosphate content was then determined in an aliquot.

The results of this part of the study are summarized in Table 2.

3. Mixed Oral Microbial Growth.—Brain-heart infusion broth was inoculated with mixed human saliva. After 72 hours of incubation, the following reaction mixture was prepared: 20 ml. brain-heart infusion growth; 2 ml. 0.4 M NaF; 2ml. 0.1 M $MgCl_2$; and 2 ml. 5 per cent glucose.

An aliquot was deproteinized with an equal volume of 10 per cent TCA, and the

orthophosphate was determined. The remainder was incubated for 24 hours at 38° C., when the orthophosphate concentration of an aliquot was again determined.

An aliquot was treated with an equal volume of pyrophosphatase enzyme from erythrocytes[3] and incubated for 1 hour at 38° C., when the orthophosphate concentration was again determined on a deproteinized aliquot.

The results of this part of the study are summarized in Table 3.

b) Decalcification of enamel, dentin, and cementum powder by pyrophosphate accumulated in salivary fermentation mixtures.—The general procedure for this part of the study was as follows: fermentation mixtures of either whole saliva, salivary debris,

TABLE 2

PYROPHOSPHATE ACCUMULATION BY SALIVARY DEBRIS

	MG. PER 100 ML. (RANGE) ORIGINAL SALIVA	
	Boiled	Active
Initial ortho-P	100	100
Final ortho-P	100	61– 72
Ortho-P regained by acid hydrolysis	0	24– 33
Pyrophosphate accumulated	0	157–188

TABLE 3

PYROPHOSPHATE ACCUMULATION BY MIXED ORAL MICROBIAL GROWTH

	MG. PER 100 ML. (RANGE) ORIGINAL MEDIA	
	Boiled	Active
Initial ortho-P	200	200
Final ortho-P	200	137–154
Ortho-P regained by pyrophosphatase action	0	46– 61
Pyrophosphate accumulated	0	262–338

or mixed microbial growth from whole saliva in brain-heart infusion broth were prepared as in section *a* above, to contain orthophosphate, magnesium ions, NaF, and glucose. The mixtures were incubated for 24 hours. The incubation mixtures were then adjusted with dilute NaOH to a pH of 7.0 and heated in a boiling water bath for 15 minutes, to inactivate them.

Half of each mixture was treated with a potent solution of yeast pyrophosphatase enzyme preparation, made according to the method of Bailey and Webb.[4] This was done to hydrolyze any accumulated pyrophosphate to orthophosphate and served as a control. Orthophosphate analysis before and after pyrophosphatase treatment was used as a measure of the amount of pyrophosphate that was present in the incubation mixtures.

Both portions of each preparation were then divided into three parts. Powdered enamel was added to one, powdered dentin to another, and powdered cementum to the third. These tooth fractions were prepared by separating powdered whole teeth with the Manly-Hodge flotation technique.

Each mixture was shaken in a rotary shaker for 96 hours, after which each fraction was acidified with HCl, heated in boiling water, and analyzed for its calcium content. The difference between the calcium concentrations in the samples treated and not treated with pyrophosphatase represents the tooth substance decalcified by accumulated pyrophosphate.

The results from this portion of the study are summarized in Table 4.

TABLE 4

DECALCIFICATION OF TOOTH SUBSTANCE BY PYROPHOSPHATE ACCUMULATED IN FERMENTATION

Tooth Substance (200-Mesh Powder)	Mixtures at pH 7.0								
	Mg/100 Ml			Mg Ca/100 Ml*					
	Pyrophosphate in Mixture			Treated with Pyrophosphatase			Not Treated with Pyrophosphatase		
	S†	D†	M†	S	D	M	S	D	M
Enamel.....	11	165	295	0	0	0	6– 8	14–26	18–35
Dentin.....	11	165	295	0	0	0	5– 8	11–27	19–32
Cementum...	11	165	295	0	0	0	4–11	9–34	17–29

* Increase in calcium concentration after shaking 96 hours with tooth substance.
† S = whole saliva; D = salivary debris; M = microbial mixture.

DISCUSSION

Tables 1–3 show that fermentation mixtures of oral microörganisms can accumulate pyrophosphate. The mixtures contained sodium fluoride to inhibit pyrophosphatase enzyme, which is also present in the fermentation mixture. If the lytic enzyme were not inhibited, little pyrophosphate would accumulate. Other pyrophosphatase inhibitors, such as hydroxylamine, beryllium salts, or iodoacetate, have also been employed in other studies with similar results. We chose to use NaF in the current work because of its obvious importance in dental investigations. The addition of equal volumes of the potent pyrophosphatase preparations lowers the inhibitor concentration below an effective level. The treatment of the fermentation mixtures with potent pyrophosphatase from erythrocytes confirms the supposition that the orthophosphate loss primarily represents pyrophosphate accumulation.

Table 4 shows that the pyrophosphate accumulated in the incubation mixtures is capable of decalcifying enamel, dentin, and cementum powder. It must be emphasized that pyrophosphate must be hydrolyzed (with HCl in our case) before determining calcium with the oxalate method because calcium pyrophosphate does not give a calcium oxalate precipitate.

It must be realized that, while these studies on pyrophosphate accumulation are under artificial conditions, in that Mg ions as well as NaF were deliberately added to demonstrate the desired results, normal fermenting salivary debris can also accumulate pyrophosphate.

A measurable amount of pyrophosphate was found in salivary debris from patients exhibiting clinical tooth erosion. None was demonstrable in debris from salivas of erosion-free mouths. These preliminary findings are shown in Table 5.

A larger number of clinical patients will be investigated, as they present themselves.

It is evident from the foregoing that a search for biological or chemical factors in human salivas which either facilitate pyrophosphate accumulation or inhibit its hydrolysis should prove profitable in helping to understand the clinical problem of erosion.

TABLE 5

PYROPHOSPHATE FROM ORAL DEBRIS RELATED TO CLINICAL EROSION

Cases	Mg. Pyrophosphate in Debris per 100 Ml. Saliva
Erosion (7)	43–67
Non-erosion (10)	0

SUMMARY

1. Human saliva, salivary debris, and mixed cultures can accumulate pyrophosphate under the conditions of this study.

2. The concentrations of pyrophosphate accumulated in fermentation mixtures are capable of decalcifying tooth substances.

3. Pyrophosphate accumulation by salivary debris may be a factor in the etiology of clinical dental erosion.

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Partial Substitution of Hexitols for Sucrose and Dextrin in Caries-producing Diets

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The hexitols, sorbitol and mannitol, have been used by bacteriologists for many years as test substrates in systematic fermentation trials for the classification and identification of microörganisms. Various strains of *Lactobacillus acidophilus* have been reported to have some ability to ferment sorbitol and mannitol.[1–3] Later, more exhaustive comparisons were made on the ability of a wide variety of strains of oral microörganisms to ferment sorbitol, glycerol, dextrose, and sucrose.[4–6] Numerous strains of common oral microörganisms were able to ferment sorbitol. However, routinely when fermentation of sorbitol occurred, it was found that the rate of acid production was slow, the total amount of acid produced was low, the final pH of the culture medium was rarely low enough to be capable of causing enamel decalcification, and the conditions for fermentation had to be optimal. Each microörganism that had any ability to ferment sorbitol could ferment dextrose or sucrose more rapidly than sorbitol. Furthermore, no greater ability to ferment sorbitol could be acquired readily.[6]

In vivo tests have been conducted in human subjects, where the pH of carious lesions was determined before and after sorbitol and sucrose rinses.[7, 8] In both studies the pH values after the sorbitol rinse did not decrease, in contrast with 5 minutes after the sucrose rinse, when readings of 6.0 or lower were observed. In one study[8] small increases in the pH values were observed after the sorbitol rinse instead of decreases; in addition, when a sucrose rinse was given 1 hour after a sorbitol rinse, the depression in pH values was not so great as when the sucrose rinse was used without the preliminary sorbitol rinse.

These studies suggested the need for trials with caries-susceptible animals to determine whether the partial replacement of sucrose or dextrin by sorbitol or mannitol in the caries-producing diet would alter the initiation and progression of carious lesions. In order for adequate tests to be conducted, relatively large amounts of sorbitol and mannitol would have to be incorporated into the diet; in so doing, extensive diarrhea could be expected to be produced in the experimental subjects. In addition, sorbitol—and mannitol to an even greater extent—are slowly absorbed from the intestinal tract and are not completely metabolized by the rat.[9–13] While these factors mitigate against an ideally designed and executed series of experiments, they were not believed to be sufficient to obscure the interpretation of the results from such experiments.

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EXPERIMENTAL METHODS

Three experiments were conducted to test the influence of partial substitution of sorbitol or mannitol for sucrose and dextrin in the diet upon the initiation and progression of carious lesions. The subjects used were offspring of the Harvard caries-susceptible strain of rats; the breeding animals of this strain have been maintained without interruption for several generations on purified diet 2700 to continue a uniform and predictable dietary background throughout tooth development and maintenance. For these experiments the rats were weaned at 21 days of age and maintained for 7–14 days on diet 2700 prior to the initiation of the experiments. In preliminary trials it had been found necessary to use rats that were 28–35 days of age because of their greater ability to withstand the extensive diarrhea caused by all the diets containing 20 per cent or more of sorbitol or mannitol. In each of the three experiments, littermates were distributed as uniformly as possible, as a means to control any inherent variation from litter to litter. Throughout the experimental periods the rats were maintained in individual wire-bottom cages with food and water supplied ad libitum. After periods of 140 days in experiments 1 and 2 and 91 days in experiment 3, the rats were sacrificed and the heads preserved in 95 per cent ethanol preparatory to skinning and evaluation of the dental caries incidence by the procedure of Shaw, Schweigert, McIntire, Elvehjem, and Phillips.[14]

The first experiment was designed to test the influence of partial substitution of sorbitol for sucrose in a caries-producing diet on the ability of the rat to survive and grow and on the initiation and progression of carious lesions. This experiment was composed of 10 groups of subjects which were fed several modifications of our basal caries-producing diet 700[15] which are presented in Table 1. The experimental design is given in the first three columns of Table 2. The rats in the first group served as controls and received ration 700. The rats in the second group received ration 711, which is identical with ration 700 except that 200 of every 670 gm. of sucrose were replaced by an equal weight of powdered crystalline sorbitol.* The subjects in the third group received ration 2711, which is ration 711 to which 15 per cent Cellu flour has been added. The fourth group received ration 713, which is identical with ration 700 except that 450 of every 670 gm. of sucrose were replaced by sorbitol. Groups 5 and 6 received rations 2713 and 3713, respectively, which are the same as ration 713 except that 15 per cent and 45 per cent Cellu flour have been added. In the seventh and eighth groups, efforts were made to determine whether the sorbitol levels could be increased as the rats grew older, with less penalty than the younger rats incurred. In group 7, ration 700 was fed for 3 weeks, followed by 11 weeks on ration 713, followed by 6 weeks on carbohydrate-free ration 2717, in which all sucrose had been replaced by sorbitol and a 15 per cent Cellu flour supplement had been included. In group 8, ration 711 was provided for 3 weeks, followed by ration 2713 for 11 weeks and ration 2717 for the remaining 6 weeks. In the ninth group, ration 4700 was fed, which is equivalent to ration 700 except that a 100 per cent supplement of Cellu flour was added. In group 10, ration 4700 was fed for 3 weeks to accustom the rats to handling very large amounts of undigestible material, followed by ration 2713 for 11 weeks and ration 2717 for 6 weeks.

* Atlas Powder Company, Wilmington, Delaware.

The design of experiment 2 is shown in the first three columns of Table 3 and is identical with that of experiment 1 except that a comparable series of diets was used in which the same amounts of powdered mannitol* instead of sorbitol were incorporated in substitution for sucrose.

The third experiment was designed to test the partial substitution of sorbitol or mannitol for dextrin in a series of diets in which all the sucrose had been replaced by dextrin. This change in design was made in the belief that the lower caries-initiating

* Atlas Powder Company, Wilmington, Delaware.

TABLE 1

COMPOSITION OF RATIONS IN GRAMS

Ration No.	Sucrose	Dextrin	Sorbitol	Mannitol	Vitaminized Casein*	Vitaminized Corn Oil*	Salt Mixture*	Whole Liver Substance	Cellu Flour
700	670	.....	.....	.....	240	50	40	40	.....
2700	670	.....	.....	.....	240	50	40	40	150
4700	670	.....	.....	.....	240	50	40	40	1,000
711	470	.....	200	.....	240	50	40	40	.....
2711	470	.....	200	.....	240	50	40	40	150
713	220	.....	450	.....	240	50	40	40	.....
2713	220	.....	450	.....	240	50	40	40	150
3713	220	.....	450	.....	240	50	40	40	450
2717	.....	.....	670	.....	240	50	40	40	150
712	470	.....	.....	200	240	50	40	40	.....
2712	470	.....	.....	200	240	50	40	40	150
714	220	.....	.....	450	240	50	40	40	.....
2714	220	.....	.....	450	240	50	40	40	150
3714	220	.....	.....	450	240	50	40	40	450
2718	.....	.....	.....	670	240	50	40	40	150
2720	.....	670	.....	.....	240	50	40	40	150
2721	.....	470	200	.....	240	50	40	40	150
2723	.....	220	450	.....	240	50	40	40	150
2722	.....	470	.....	200	240	50	40	40	150
2724	.....	220	.....	450	240	50	40	40	150

* *J. D. Res.*, **26**:47, 1947.

TABLE 2

INFLUENCE OF PARTIAL SUBSTITION OF SORBITOL FOR SUCROSE ON EXPERMENTAL DENTAL CARIES

Group No.	Ration	No. Rats Begun	No. Survivors	Av. Weight Gain of Survivors (Gm.)		Av. No. Carious Molars	Av. No. Carious Lesions	Av. extent of Carious Lesions
				♂	♀			
1	700	5	4	255	156	9.5	21.8	89+
2	711	5	3	190	189	8.0	16.0	59+
3	2711	6	4	250	136	7.8	15.8	60+
4	713	10	0	.....	.....	.....	.....	.....
5	2713	10	2	73	69	8.0	14.0	48+
6	3713	10	1	.....	39	6.0	12.0	41+
7	700–713–2717	5	3	81	46	9.0	16.3	63+
8	711–2713–2717	9	8	70	23	6.4	12.3	46+
9	4700	6	4	145	91	8.0	15.3	86+
10	4700–2713–2717	4	4	98	59	6.8	9.5	57+

influence of dextrin than sucrose would be less likely to obscure any changes in dental caries experience that occurred as a result of the inclusion of sorbitol or mannitol in the diet. This experiment consisted of 8 groups of rats and lasted for 91 days. The first group of rats received ration 2700. The rats in the second group can be considered as the controls against which the others will be compared. They received ration 2720, which is the same as ration 2700 except that all sucrose had been replaced by the same weight of dextrin. The subjects in group 3 received ration 2721, which was similar to ration 2720 except that 200 of every 670 gm. of dextrin were replaced by sorbitol. In group 4, the rats were fed ration 2723, which was similar to ration 2720 except that 450 of every 670 gm. of dextrin were replaced by sorbitol. The rats in group 5 were fed ration 2721 for 4 weeks followed by ration 2723 for 9 weeks. The rations fed

TABLE 3

INFLUENCE OF PARTIAL SUBSTITUTION OF MANNITOL FOR SUCROSE ON EXPERIMENTAL DENTAL CARIES

Group No.	Ration	No. Rats Begun	No. Survivors	Av. Weight Gain of Survivors (Gm.)		Av. No. Carious Molars	Av. No. Carious Lesions	Av. Extent of Carious Lesions
				♂	♀			
1.....	700	5	5	221	144	7.6	16.2	62+
2.....	712	6	4	252	129	7.5	14.0	46+
3.....	2712	5	2	184	63	7.5	12.5	41+
4.....	714	10	1	.....	90	4.0	8.0	32+
5.....	2714	10	2	.....	97	4.0	5.5	15+
6.....	3714	10	0	.....	.....	.....	.....	.....
7.....	700–714–2718	5	5	76	57	6.2	12.0	43+
8.....	712–2714–2718	9	6	115	48	5.3	8.4	26+
9.....	4700	5	5	147	94	8.6	16.7	68+
10.....	4700–2714–2718	5	3	.....	69	5.0	8.3	25+

groups 6, 7, and 8 were identical with those fed groups 3, 4, and 5, respectively, except that mannitol was used instead of sorbitol.

RESULTS

The data for experiments 1 and 2 are summarized in Tables 2 and 3, respectively. Relatively small numbers of rats were used in these experiments, as they were preliminary trials to determine the levels of sorbitol and mannitol that could be tolerated by our strain of caries-susceptible rats for the length of assay period required. Moderate to extensive diarrhea was observed in almost every rat that consumed a diet containing 20 per cent or more of sorbitol or mannitol. From these data it can be concluded that levels of 20 per cent sorbitol or mannitol, as in rations 711, 2711, 712, and 2712, can be tolerated reasonably well for prolonged periods by rats when begun at 28–35 days of age. Although about one-third of the rats on the 20 per cent levels died in the early days of the experiment, the remainder grew well and remained reasonably healthy, though diarrhea was moderate to extensive throughout the experimental period. In all groups fed these four rations, modest reductions in tooth decay were observed as compared with the control group 1 in each experiment.

When the sorbitol and mannitol levels were increased to 45 per cent, as in diets

713, 2713, 3713, 714, 2714, and 3714, 80–100 per cent of the rats died in the early weeks of the experiments. The caries experience among the scattered survivors from these groups tended to be slightly lower than for the rats in the groups fed diets containing 20 per cent sorbitol or mannitol. Increases in the amount of the Cellu flour supplement from 0–15 to 45 per cent in the attempt to control the diarrhea did not appear to aid in the survival of these rats. Beginning the diets containing 45 per cent sorbitol or mannitol at later ages, as in group 7 of each experiment, increased the rate of survival, although the rats grew very poorly throughout the experimental period. Stepwise increases in the amounts of sorbitol and mannitol, as attempted in group 8 of each experiment, tended to permit increased survival. In groups 7 and 8 the surviving rats after 14 weeks on lower levels of sorbitol or mannitol were able to exist for 6 weeks on carbohydrate-free diets 2717 and 2718, which contained 67 per cent of one of the hexitols. Modest reductions in caries resulted from these procedures.

The addition of 100 per cent Cellu flour to the cariogenic diet in group 9 of each experiment did not alter the initiation and progression of carious lesions from that observed among the control rats in group 1, although their rate of growth was materially reduced. The beginning of the 45 per cent sorbitol and mannitol diets in group 10 of each experiment after a period of accommodation to diet 4700 containing the 100 per cent Cellu flour supplement permitted good survival. However, part or all of this effect may have been a result of the increased age and weight of the rats, as in group 7 of experiments 1 and 2. The rats in group 10 of both experiments had substantially less caries than did the control rats. The carious lesions in the rats of groups 7, 8, and 10 of both experiments where carbohydrate-free diets 2717 and 2718 had been fed for the 6 weeks preceding sacrifice were stained and hard, with no evidence of active progression of the carious process.

No statistical comparisons of these data are justified in view of the small number of rats. However, from inspection of the data in Tables 2 and 3 it can be concluded that wherever 20 per cent or more of sorbitol or mannitol was used in substitution for sucrose in the diets fed the groups of rats in these experiments, modest and consistent reductions in tooth decay were observed. These data strongly suggest that neither sorbitol nor mannitol supports the caries process under the conditions of these experiments. The amounts of sucrose in all diets except 2717 and 2718 are sufficient to promote a major rate of initiation and progression of carious lesions. This caries-promoting effect of the sucrose could overshadow any minimal initiation and progression caused by the hexitols.

The data from the third experiment are presented in Table 4. The rats in group 1 maintained on the sugar-containing cariogenic diet 2700 grew well and had a typical incidence of carious lesions. The control rats in group 2, which received diet 2720, where sucrose had been replaced by dextrin, grew almost as well as those fed diet 2700 and had, on the average, 11–23 per cent less caries. Moderate to extensive diarrhea was observed throughout the experimental period in practically every rat in groups 3–8. In group 3, where ration 2721 with approximately 20 per cent sorbitol was fed, the rats grew poorly, 6 of the 17 died, and significant reductions in caries were observed, varying from 43 per cent for the average number of carious molars to 53 per cent for the average extent of carious lesions. In group 4, where ration 2723 containing approximately 45 per cent sorbitol was fed, only 2 of 17 rats survived the

full experimental period. These rats grew very slowly and had experienced an incidence of dental caries very little lower than the rats fed ration 2720. In view of the few survivors, no fair comparison can be made with respect to the influence of diet 2723 on experimental dental caries. In group 5, where ration 2721 was fed for 4 weeks followed by ration 2723 for 9 weeks, 9 of the 17 rats survived and grew relatively slowly. These rats had amounts of tooth decay which differed highly significantly from the rats fed ration 2720 and which were slightly, but insignificantly, lower than for the rats fed ration 2721 throughout the experimental period.

In group 6, where the rats were fed ration 2722 containing 20 per cent mannitol, 16 of the 17 survived and grew moderately well, although not so well as those fed ration 2720. These rats had less tooth decay than those fed ration 2720, with the re-

TABLE 4

INFLUENCE OF PARTIAL SUBSTITUTION OF SORBITOL AND MANNITOL FOR DEXTRIN ON EXPERIMENTAL DENTAL CARIES

Group No.	Ration No.	No. Rats Begun	No. Survivors	Av. Weight Gain of Survivors (Gm.)		Av. Weight of Cecum and Contents (Gm.)	No. Carious Molars			No. Carious Lesions			Extent of Carious Lesions		
				♂	♀		Av.	SEM*	CR†	Av.	SEM*	CR†	Av.	SEM*	CR†
1.....	2700	18	18	199	117	3.4	7.3	0.5	1.4	13.3	1.3	1.2	44+	5+	1.3
2.....	2720	17	15	185	105	3.1	6.1	.7	4.0	10.9	1.5	3.8	34+	6+	4.0
3.....	2721	17	11	119	82	6.3	3.5	.8	2.5	5.4	1.6	2.5	16+	5+	2.3
4.....	2723	17	2	89	38	8.0	5.5	...	2.9	9.5	...	3.5	29+	...	3.3
5.....	2721–2723	17	9	104	66	10.9	3.0	.8	3.3	3.9	1.3	3.4	10+	4+	3.5
6.....	2722	17	16	134	92	7.4	4.5	.7	1.6	6.8	1.4	2.0	19+	5+	1.9
7.....	2724	16	1	142	.....	17.0	4.0	...	0.7	5.0	...	1.8	13+	...	1.7
8.....	2722–2724	17	11	131	62	10.9	5.4	0.7		7.5	1.2		22+	4+	

* SEM = Standard error of mean.

† CR = Critical ratio. The critical ratio is the ratio of the difference between two means to the standard error of the difference between the means. Wherever the critical ratio is less than 2.0, the difference between the means is considered to be statistically insignificant; when from 2.0 to 2.9, moderately significant; when 3.0 or higher, highly significant.

ductions varying from 26 per cent for the average number of carious molars to 44 per cent for the average extent of carious lesions. These reductions were consistent but were just below the borderline of statistical significance. In group 7, where the rats were fed ration 2724 containing 45 per cent mannitol, only 1 rat survived, which had a very low dental caries experience. In group 8, where the rats were fed ration 2722 followed by ration 2724, 11 of the 17 survived; the males grew fairly well, although the females did much more poorly. These rats had about the same amount of tooth decay as those in group 6 fed ration 2722. Although there were consistent reductions in dental caries experience from the rats fed ration 2720, these reductions again were just below the borderline of statistical significance.

The cecum and its contents were weighed together at the time the rats in experiment 3 were sacrificed. In the rats of groups 1 and 2 without sorbitol or mannitol in the diet, the cecum and its contents weighed an average of 3.4 and 3.1 gm., respectively. The cecum in all rats in groups 3–8 was enlarged. The rats in groups 3 and 6, where

the diets containing 20 per cent sorbitol and mannitol were fed, respectively, had average weights for the cecum and its contents of 6.3 and 7.4 gm. Little data are available for groups 4 and 7 because of the few survivors: the cecum and contents of the two survivors in group 4 averaged 8.0 gm., while the cecum and contents of the one survivor in group 7 weighed 17.0 gm. In groups 5 and 8, where low levels of sorbitol were followed by high levels, the average weight of the cecum and its contents was 10.9 gm. for each group.

DISCUSSION

From these data the conclusion can be drawn that neither sorbitol nor mannitol supported the initiation and progression of carious lesions at a rate comparable to sucrose or dextrin under the experimental conditions in these trials. If these hexitols supported the caries process at all, their influence must have been completely obscured by the much greater influence of the amounts of the carbohydrates in the various diets. In those few experimental subjects to which carbohydrate-free diets containing 67 per cent sorbitol or mannitol were fed during the last 6 weeks of experiments 1 and 2, the impression was gained that caries activity had ceased in the lesions which had been initiated in the first 14 weeks on experiment and which were undoubtedly active at the time the feeding of the carbohydrate-free diets was begun.

Although sorbitol and mannitol have little or no ability to support the caries process, their use in human nutrition must be limited by reason of their limited tolerance. For example, Ellis and Krantz[10] found that very soft watery stools were produced by the daily consumption of 10–20 gm. of mannitol, 20–30 gm. of sorbitol, or 50 gm. of crystalline sorbitol by 12 normal individuals. In other studies, Peters and Lock[16] fed 25 gm. of sorbitol per day in 2 doses to 84 volunteers for 1 week. Discomfort and laxation were experienced by 5 subjects, 2 of whom did not complete the week. The remainder encountered no significant problem except for a slight tendency to flatulence after sorbitol ingestion. The amounts of sorbitol used as a sweetening agent and humectant in various confections are likely to be less than enough to cause problems unless excessive amounts of the confections are consumed. On the basis of the present experiments, it would seem likely that the use of sorbitol instead of sucrose or glucose in these confections would reduce their caries-producing ability.

SUMMARY

Three experiments were conducted with caries-susceptible white rats in which sorbitol or mannitol were used in partial substitution in cariogenic diets for sucrose or dextrin. Wherever 20 per cent or more of sorbitol or mannitol was present in the cariogenic diets, moderate but consistent reductions in caries experience were noted. Although an absolute statement cannot be made about the ability of sorbitol and mannitol to initiate or maintain the caries process because of the need for the simultaneous presence of some caries-initiating carbohydrates in the diets, it appears that these hexitols have little or no capacity to promote the caries process.

The tendency of sorbitol and mannitol to produce diarrhea among the experimental animals prohibited the feeding of high amounts for prolonged periods or their complete replacement for carbohydrates except under unusual circumstances. However, 20 per cent of the hexitols could be tolerated in the diets of these rats for periods of up

to 140 days. Higher amounts could be tolerated, provided that the rats were older at the beginning of the feeding trials or that they had been accustomed to lower levels previously.

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Cementum of Periodontally Diseased Teeth from India

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In a study of the thickness of cementum in relationship to age, Hürzeler and Zander[1] found that at all ages the cementum thickness in teeth from the United States was less in teeth with diseased periodontium* than in teeth with healthy periodontium.† Therefore, it seemed of interest to determine whether this trend could also be found in periodontally diseased teeth from India. Unfortunately, periodontal disease seems to be so universal in India that we were unable to find a control group of Indian teeth having no periodontal disease.

TABLE 1

NUMBER OF TEETH USED IN THIS STUDY BY TYPE AND AGE GROUP

Age Group	Tooth Group								
	Upper Central Incisors	Lower Central Incisors	Upper Lateral Incisors	Lower Lateral Incisors	Upper Canines	Lower Canines	Upper Bicuspids	Lower Bicuspids	Total
21–30	5	2	2	2	.....	.....	1	4	16
31–40	7	5	1	2	6	3	1	2	27
41–50	3	3	1	1	4	3	5	6	26
Over 50	7	4	2	5	7	6	3	6	40

MATERIALS AND METHODS

One hundred and nine single-rooted teeth from the Hindu population were used for this study.‡ The teeth were received in formalin, being separated in age groups twenty-one to thirty, thirty-one to forty, forty-one to fifty, and over fifty years of age of the patient at the time of extraction. They had been removed from patients with periodontal disease, and no patient had contributed more than four teeth.§ The distribution of the teeth, according to age group and type of tooth, is shown in Table 1.

The teeth were decalcified in 5 per cent nitric acid and imbedded in celloidin. Hori-

This study was conducted under U.S. Army Contract DA-49-007-MD-857 at the Eastman Dental Dispensary.

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* Referred to henceforth as "Diseased America Teeth."

† Referred to henceforth as "Healthy America Teeth."

‡ The teeth were collected and sent to us by Dr. Vimla Sud, dental surgeon, Safdarjang Hospital, New Delhi, India.

§ Referred to henceforth as "Diseased India Teeth."

zontal sections, 20 μ thick, were made at 80-μ intervals from the apex to the cemento-enamel junction. After the sections had been stained with hematoxylin and eosin, the average cementum thickness was determined by the method of Zander and Hürzeler,[2] i.e., by dividing the volume of the cementum by the surface area of the root. Measurements were made for four areas of each tooth: the "total root"; the "apex," comprising the first ten sections or apical 0.8 mm. of the root; the "crown end," comprising the first section in which the cemento-enamel junction appeared to the last section which showed any cementum; and the "middle," that is, the section which is equidistant between the "apex" and the "crown end."

TABLE 2

AVERAGE CEMENTUM THICKNESS (MM.) ± STANDARD ERROR

	Age Group	Av. Age	No. of Teeth	"Total Root"	"Apex"	"Middle"	"Crown End"
Diseased India teeth	21–30	.....	16	0.084±0.007	0.190±0.028	0.076±0.006	0.044±0.003
	31–40	.....	27	.092± .006	.254± .033	.081± .005	.041± .002
	41–50	.....	26	.093± .006	.239± .027	.089± .008	.046± .002
	Over 50	.....	40	.121± .007	.268± .027	.134± .009	.035± .003
Diseased America teeth	21–30	29.4	10	.092± .010	.219± .024	.091± .010	.058± .003
	31–40	35.3	13	.076± .004	.198± .018	.079± .004	.049± .002
	41–50	46.3	23	.133± .017	.312± .024	.144± .003	.063± .004
	51–73	60.3	37	.147± .006	.330± .027	.162± .008	.070± .003
Healthy America teeth	21–30	25.6	34	.111± .005	.283± .017	.115± .006	.082± .005
	31–40	36.2	38	.137± .003	.352± .006	.161± .006	.100± .003
	41–50	44.0	37	.151± .007	.434± .028	.157± .009	.108± .008
	51–76	59.5	70	0.215±0.009	0.585±0.034	0.238±0.011	0.128±0.004

RESULTS

In Table 2 the average cementum thicknesses in the different root areas of the diseased India teeth are compared with those found in corresponding age groups in the previous study of diseased America teeth[1] and healthy America teeth.[2]* The average cementum thickness of the diseased India teeth for all age groups except the one from thirty-one to forty years was less than that of the diseased America teeth.

The differences are shown in the four graphs (Figs. 1–4), on which points representing the average cementum thickness of the diseased India teeth were superimposed on those presented in the paper "Cementum Apposition in Periodontally Diseased Teeth."[1] The graphs show both a straight and a curved line for average cementum thickness of diseased America teeth. The curved line was constructed by using a multiple regression equation involving age up to the third power and gives the best picture of average cementum thickness in relationship to age. It shows clearly a leveling-off, i.e., a diminished increase, in average cementum thickness with increasing age. The straight line was constructed only for comparison with the healthy America teeth, where average cementum thickness actually increased in a straight-line relationship with age. The graphs demonstrate that in all four root areas there was less cementum

* The results from the previous papers are reprinted in this table because they were originally published in a different journal.[1]

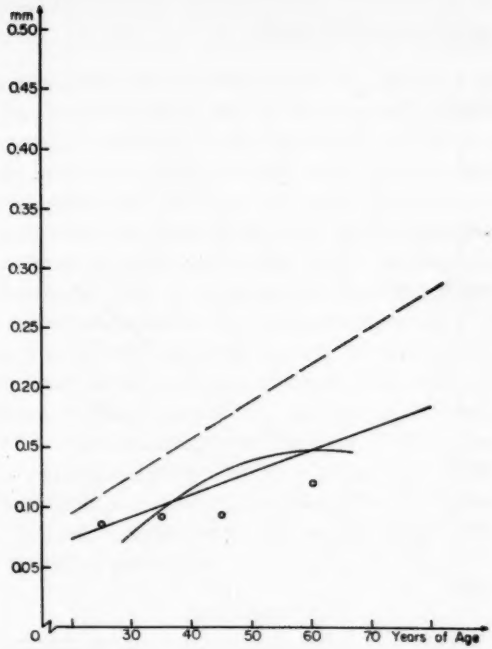

FIG. 1.—Average cementum thickness for "total root." —— —— ——, healthy America teeth; ————, diseased America teeth; 0, diseased India teeth.

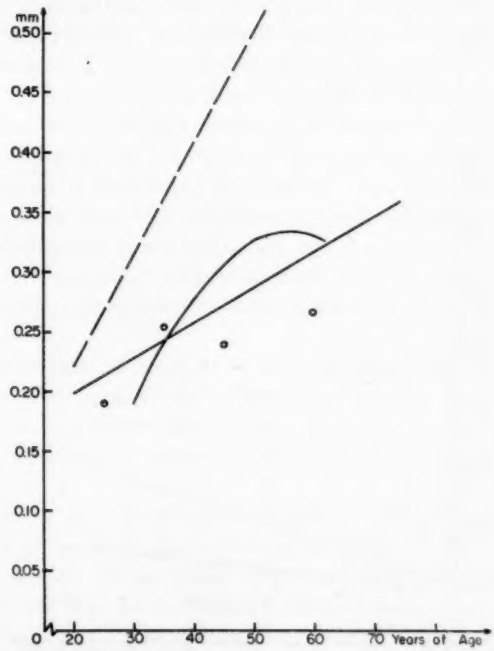

FIG. 2.—Average cementum thickness at "apex." —— —— ——, healthy America teeth; ————, diseased America teeth; 0, diseased India teeth.

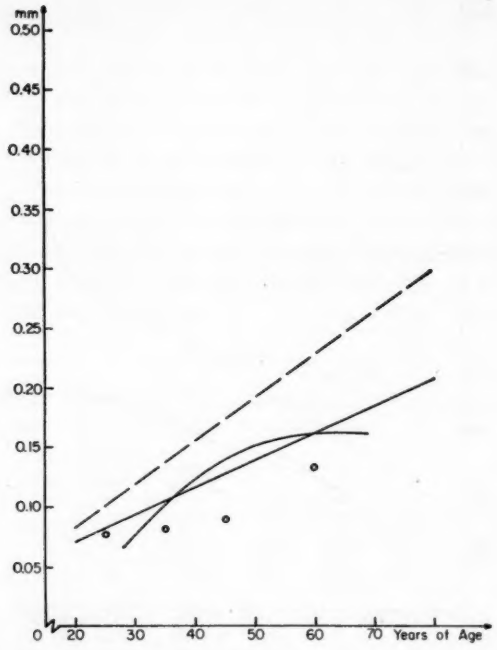

FIG. 3.—Average cementum thickness at "middle." — — —, healthy America teeth; ———, diseased America teeth; 0, diseased India teeth.

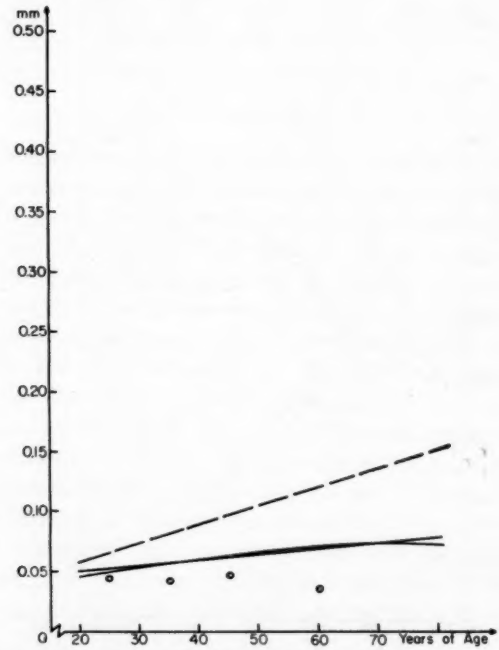

FIG. 4.—Average cementum thickness at "crown end." — — —, healthy America teeth; ———, diseased America teeth; 0, diseased India teeth.

on the diseased India teeth than on the diseased America teeth or the healthy America teeth. The graphs also show that there was in the diseased India teeth very little increase in average cementum thickness during life except for the areas "total root" and "middle," where in the over-fifty age group there was a definite increase.

Standard errors were calculated for each age group and tooth area of the diseased India teeth and are comparable to those of the teeth from the United States (Table 2). The standard error is relatively high only at the "apex" because of the great variation in cementum thickness in this area. In constructing the original graphs it was possible to apply a multiple regression equation for the teeth from the United States because their exact age at the time of extraction was known. Of the teeth from India, only the age group was known, and an average age of twenty-five, thirty-five, forty-five, and sixty years was assumed for the respective age groups. At this assumed average age a point was plotted for the average cementum thickness. With the use of this method a confidence interval of twice the standard error on either side of the average should be applied. A significant difference between average cementum thickness of the diseased India teeth and the diseased America teeth and healthy America teeth appeared upon application of these statistical principles.

DISCUSSION

Some interesting differences were noted between the diseased India teeth and those previously studied. In the diseased India teeth the average cementum thickness remained virtually the same during life, except for "total root" and "middle," where in the over-fifty age group there was a marked increase. This was contrary to the diseased America teeth, in which Hürzeler and Zander[1] found a leveling-off of cementum apposition with age to be typical. This increase could be explained by considering average cementum thickness as a function of age and the degree of periodontal disease. While cementum gets thicker with age, less cementum is found in connection with a higher degree of periodontal disease; or, conversely, if in a certain age group more cementum is found, less periodontal disease must be assumed. This probably holds true for the diseased India teeth in the over-fifty age group. If teeth have a severely diseased periodontium, they are usually lost at a much earlier age than sixty. Therefore, the diseased India teeth of this last age group were, compared with the other age groups, a selection of teeth with better periodontal structures and thus showed an increase in average cementum thickness.

It is understandable that an increase in average cementum thickness in the over-fifty age group cannot be seen at the "crown end" because in this area a pocket is formed very early in life, preventing further cementum apposition. Following this pocket formation, only a *loss* of cementum can occur, due to erosion or other kinds of trauma.

The plotted points for the "apex" of the diseased India teeth are slightly irregular, with a wide confidence interval due to a wide variation in the cementum thickness. Therefore, nothing specific can be stated concerning this area other than the general finding of less cementum than on the diseased America teeth.

In attempting to interpret the significance of "average cementum thickness," it must be borne in mind that it may be influenced by several factors besides the apposition stressed by Hürzeler and Zander.[1] These are the frequency and amount of resorption

and repair, the presence of defects or tears, and erosion. That resorption may influence average cementum thickness is supported by the finding of Harvey and Zander,[3] whose study included teeth used in this study and in the previous ones.[1,2] They found five times more resorption in the diseased India teeth than in the healthy America teeth. However, this cannot be the complete explanation, because the maximum resorption observed in any age group of diseased India teeth involved only 6.8 per cent of the circumference, and, even if this resorption had extended through the entire thickness of the cementum, it could not account for the 50 per cent less cementum found in the present investigation. Therefore, other factors which influence average cementum thickness must be more important.

At present, no data are available on the occurrence of cementum defects; we merely gained the impression that there were many more such defects on the roots of the diseased India teeth than on those in the other groups. If specific information on the occurrence of cementum defects were available, a sounder conclusion could be drawn in respect to the reasons for the differences in average cementum thickness between the three groups studied. It is worth suggesting that these differences are more likely to be the result of the frequent occurrence of cementum defects or tears due to changes in the *quality* of the cementum than of differences in rate, speed, or lack of cementum apposition. Futher investigations are necessary to substantiate this theory.

SUMMARY

Measurements of cementum thickness were made on 109 single-rooted teeth from India. The average cementum thicknesses in different root areas of these teeth were compared with those found in previous studies of healthy and periodontally diseased teeth from the United States. There appeared a significant difference between the average thickness of the cementum in these three groups. There was less cementum on the teeth from India than on those from the two American groups.

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Physical Properties of Elastic Duplicating Materials

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Agar has been used for a number of years in the preparation of elastic duplicating materials. These duplicating materials have been used mainly in partial denture prosthesis for the preparation of a duplicate refactory cast of the original stone cast of the patient's mouth. Since moderately large quantities of duplicating materials are used in these procedures, the low cost and reversibility of the agar materials are a definite advantage.

The mercaptan and silicone rubber elastic impression materials have not been used extensively in duplicating procedures because of their high cost and the fact that these materials, once set, are irreversible. Recently, a plastic duplicating material has been developed which is a reversible polyvinyl chloride plastic gel. Because of its chemical and physical nature, this material has elastic properties somewhat different from the agar compounds.

The agar duplicating compounds are composed primarily of agar and water but may also contain borax to increase the strength, salts to accelerate the setting of the investment, and other ingredients. Cellulose fibers, waxes, and fillers, which may be found in impression compounds, generally are omitted from duplicating compounds, since the duplicating materials are often stored in the sol state for extended periods and these ingredients tend to separate under this condition of storage. Since the duplicating compounds may be stored at temperatures of 130°–150° F., their resistance to thermal degradation is an important property.

The basic composition of the agar impression compounds is much the same as for the agar duplicating compounds. The principal difference is that the impression compounds may contain 10–15 per cent agar, while the duplicating materials may contain only 5 per cent agar and, as just indicated, need not contain waxes, fillers, and other modifiers common to impression materials. In fact, agar impression materials have been used as duplicating compounds by simply diluting the impression material with an equal amount of water.

The properties of the agar impression compounds have been studied by numerous investigators. Skinner and Kern[1] determined the linear-dimensional changes in air and subsequently in water of various agar impression compounds. They also measured the compressive properties of proportional limit, maximum flexibility, and crushing strength, as well as the plastic flow properties of these agar materials. Paffenbarger[2] reported the compressive properties of strength, deformation, set, and stiffness of a number of agar compounds. The temperature of gel formation and the impression-

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taking qualities, as measured by the present A.D.A. master model for agar hydrocolloid materials, were also determined. Luster[3] reported the effect of environmental factors of air, water, and inorganic sulfate solutions on the dimensional stability of agar gels. Phillips and co-workers[4] also measured the dimensional changes of agar gels under various conditions.

The properties of elastic duplicating materials have received little attention, with only limited information available in the literature. Margetis and Hansen,[5] however, recently studied the changes in agar duplicating materials on heating and storage by measuring changes in the pH and viscosity of the sols and the crushing times of the gels. These values indicated a gradual degradation of the agar material over a 32-day period when stored at 52° C. (125.6° F.).

TABLE 1

DUPLICATION AND IMPRESSION MATERIALS STUDIED

Code Letter	Product	Supplier
	Duplicating Compounds	
A.....	Copymaster Duplicating Material	L. D. Caulk Co.
B.....	Lastic	Durallium Products Corp.
C.....	Nobiloid	Nobilium Products, Inc.
D.....	Surgident Duplicating Compound	Surgident, Ltd.
E.....	Ticonium Special	Dental Perfection Co.
F.....	"Van R" Laboratory Duplicating Material	"Van R" Dental Products
	Impression Compounds	
G.....	Deelastic	Kerr Manufacturing Co.
H.....	Dentocoll	L. D. Caulk Co.
I.....	Surgident	Surgident, Ltd.

The present investigation represents a portion of a study being conducted on elastic duplicating materials and consists mainly of an initial comparison of the properties of duplicating materials with agar impression compounds. The physical properties of compressive strength, tear strength, stress-strain properties, per cent set, crushing time, impression qualities, and gelation temperature were measured. In addition, the properties of a number of experimental agar gels were determined, in order to examine the effect of the agar source and composition on physical properties.

Other phases of this study, to be reported at a later date, are (1) the accuracy of duplicating materials, (2) the effect of aging on duplicating compounds, and (3) the visco-elastic behavior of these materials.

MATERIALS AND METHODS

The commercial duplicating materials investigated and the suppliers are listed in Table 1, with a code letter for each material. The materials are referred to by code letter because this study is an evaluation of the general properties of elastic dupli-

cating materials, and a high or low value for any particular property does not imply that the material is a superior or inferior product. In fact, it is known that each of the materials is used routinely and gives acceptable results. All the duplicating compounds are hydrocolloid materials except material B, which is a reversible plastic gel.

Three hydrocolloid elastic impression materials were selected from the large number commercially available on the basis of general differences in physical properties. These products also are listed in Table 1 with a code letter.

Experimental agar gels were prepared from four different commercially available dental-grade agars* used in these investigations. Samples from different batches of these raw agars were obtained and the physical properties determined, in order to observe differences as a result of the source of supply. A dental-grade agar was also used to prepare experimental gels. Five lots of this type of agar† taken over an 18-month period were examined.

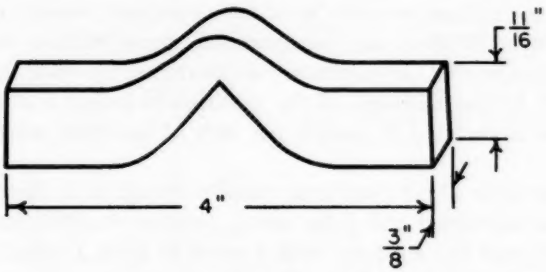

FIG. 1.—Sketch of a tear specimen

Specimens used for the determination of compressive strength, stress-strain curves, per cent set, and crushing time were cylinders ¾ inch high by ½ inch in diameter. These cylindrical specimens of agar impression or duplication compounds were prepared by liquefying them in boiling water and using specimen molds as described in A.D.A. specification No. 11 for hydrocolloid impression material, section 4.4.5.1.[6] The duplicating compounds were poured when the temperature was between 140° and 150° F. The impression compounds were tempered according to the manufacturer's directions. Duplicating material B was poured at the storage temperature of 210° F. After pouring, the specimens were stored in a humidor at 100 per cent relative humidity for 1 hour until tested.

The tear specimens used had the shape of the ASTM tear-test die C (ASTM Test D624-54).[7] The tear test has been used to evaluate rubber materials. The shape and dimensions of the specimens are indicated by the sketch in Figure 1. The mold for the tear specimens was prepared from a casting plastic, and the top and bottom of the mold were formed by glass plates. The specimens were prepared and stored as indicated for the compression specimens.

The dry strip agar was cut into small pieces, and the powdered forms were used as received. 10 gm. of agar plus 190 gm. of water were placed in half-pint mason jars.

* Japanese strip agar, Kobe #1, Portuguese agar, Type PAR 40, and Spanish agar, Type PAR 80; T. M. Duché and Sons, Inc.

† American Agar and Chemical Company.

After the agar was thoroughly wetted, the sealed jar was placed in an autoclave for 15 minutes at 265° F. After this time, the pressure was released and the agar sols allowed to cool to room temperature.

The compressive strength of the various materials was determined on the cylindrical specimens. The load required to rupture the specimen was determined, using an Instron Testing Machine. The rate of deformation during testing was varied from 1 to 20 inches/minute. It was observed that a maximum compressive strength resulted at a deformation rate of approximately 10 inches/minute, for which reason the majority of the testing was carried out at this deformation rate.

The stress-strain curve may also be obtained from the load and deformation data. The stress-strain curves were plotted from the data obtained at a deformation rate of 10 inches/minute. These curves permitted the determination of the stiffness, proportional limit, and per cent compression of the agar materials.

The tear strength of elastic duplicating and impression materials is a most important property because these materials must have sufficient strength that they may be removed from undercut areas and interproximal spaces without tearing. The load required to continue tearing the specimen was determined by using the Instron Tester. The load divided by the thickness of the specimen in inches was a measure of the tear strength and is reported in pounds per inch of notch or, more simply, pounds per inch.

The per cent set after 12 per cent strain was determined on an instrument developed by Cresson.[8] The instrument had a dial gauge sensitive to 0.001 inch, was mounted on a steady stand, and was equipped with a screw to allow a cylindrical specimen to be compressed the desired 12 per cent. The sample was compressed 12 per cent and the pressure maintained 1 minute, after which the sample was allowed to rest with no pressure for 1 minute, and the specimen height was measured. Knowing the original and final specimen length, the per cent set may be calculated.

A crushing-time test similar to that proposed by Margetis and Hansen[5] was used. The modified compression-testing apparatus described by Paffenbarger[2] was convenient for performing the crushing-time test. The cylindrical specimens were loaded with weights varying from 1,100 to 3,600 gm., and the time from the load application to the rupture of the specimen was determined in seconds. Variation in the load was necessary, since no single load could be found which would give reasonable crushing times for agar impression, duplication, and experimental materials.

The A.D.A. master model for testing hydrocolloid impression materials[6] was used to give a general evaluation of the impression qualities of the duplicating materials. This master model has five posts, two of which are cylindrical, and the other three have varying degrees of undercut. A snap removal of the master model was carried out, and improved stone was used to prepare the duplicate model.

The gelation temperatures were measured according to the directions of the A.D.A. specification No. 11 for hydrocolloid impression materials.[6] The test consists of inserting a metal tube into the impression material at various times as the material cools from a sol to a gel. The gel temperature is that temperature at which a clear impression of the inside and outside of the metal tube is obtained and none of the material clings to the walls of the tube.

RESULTS

Stress-strain properties.—The stress-strain curves were determined for all the duplicating and impression compounds listed. The values for the initial slope of the straight-line portion of the curves and the proportional limit as indicated by flattening of the curve prior to fracture are listed in Table 2. In addition, the ultimate per cent compressions also are reported in this table. The ultimate compressive strength also may be obtained from these curves, and this property will be discussed in the following section. Examples of the various types of stress-strain curves obtained are presented in Figure 2 for materials B, D, E, and I.

TABLE 2

STRESS-STRAIN PROPERTIES OF DUPLICATING AND IMPRESSION COMPOUNDS

Material	Stiffness (lb/in²)	Prop. Limit (lb/in²)	Ultimate Compression (per cent)
	Duplicating Compounds		
A	150	59	39
B	100	130	60
C	70	51	46
D	120	54	36
E	70	39	32
F	140	58	31
	Impression Compounds		
G	210	72	37
H	200	102	46
I	200	95	42

The deformation rate was 10 inches/minute, with the result that the complete stress-strain curve for any of the samples was obtained in less than 3 seconds. On the basis of unpublished flow studies, the curves represented primarily the elastic behavior of the materials, and any viscous effect was neglected. Material I was an agar impression compound and, like the other impression compounds G and H, was stiffer and had a higher proportional limit than the agar duplicating compounds. These results would be expected, since the agar duplicating compounds are more dilute than the agar impression materials. Agar duplicating compounds A, D, and F were similar in their compressive properties, while compounds C and E had lower compressive characteristics. This again would be anticipated because of the lower agar concentrations used in materials C and E. Material B was a plastic gel duplicating material and had compressive properties similar to agar duplicating compounds at low compression values. This material had considerably higher strength and ultimate compression values than did the agar types. It is important to point out that these high values are

not required of a duplicating material. It may, however, affect the length of time over which the duplicating compound may be used before the physical properties deteriorate to such an extent that the material must be discarded.

The ultimate per cent compression of the agar duplicating compounds was generally lower than for the impression compounds, but no correlation existed between these values and the other compressive properties.

The shape of the stress-strain curves was different from those reported by Paffenbarger.[2] The initial portion of the curves in Figure 2 were straight lines, but between a strain of 0.1 and 0.2 inch/inch the curves began to rise. After this rise they flattened out and finally became discontinuous when the specimens ruptured. This shape may

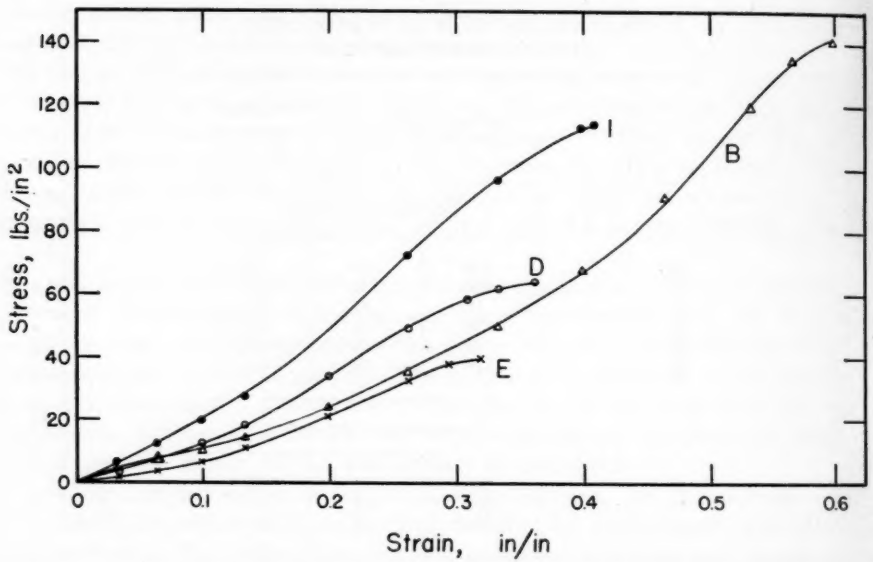

FIG. 2.—Stress-strain curves for duplicating compounds B, D, and E, and impression compound I

be explained by the fact that the specimen cross-sectional area is increasing during the test or that the stiffness of the material increases with the deformation. The stress-strain curves reported by Paffenbarger[2] were usually concave toward the strain axis. The differences in the results of the two studies may be reconciled by noting that the latter stress-strain curves were obtained by loading the specimens at the rate of 2.84 lb/in²/minute, and therefore, the tests required 10–17 minutes to complete. Considerably more viscous deformation could occur in this period of time compared to the 3 seconds or less required in the present study. Any flow taking place would result in the stress-strain curve being concave toward the strain axis. The curves shown in Figure 2, therefore, more nearly represent the behavior of the agar materials in service, since the impression is removed rapidly rather than slowly.

Compressive strength.—The compressive strengths of the duplicating compounds are shown in Figure 3, those for the agar impression compounds in Figure 4, and those for the experimental agar gels in Table 3. The compressive strengths, measured at a

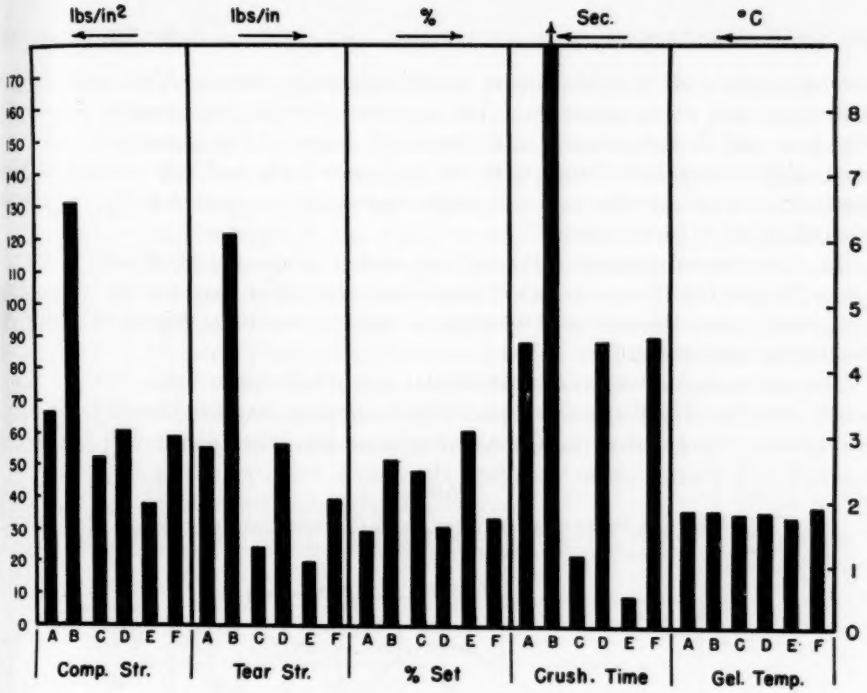

FIG. 3.—Properties of duplicating compounds

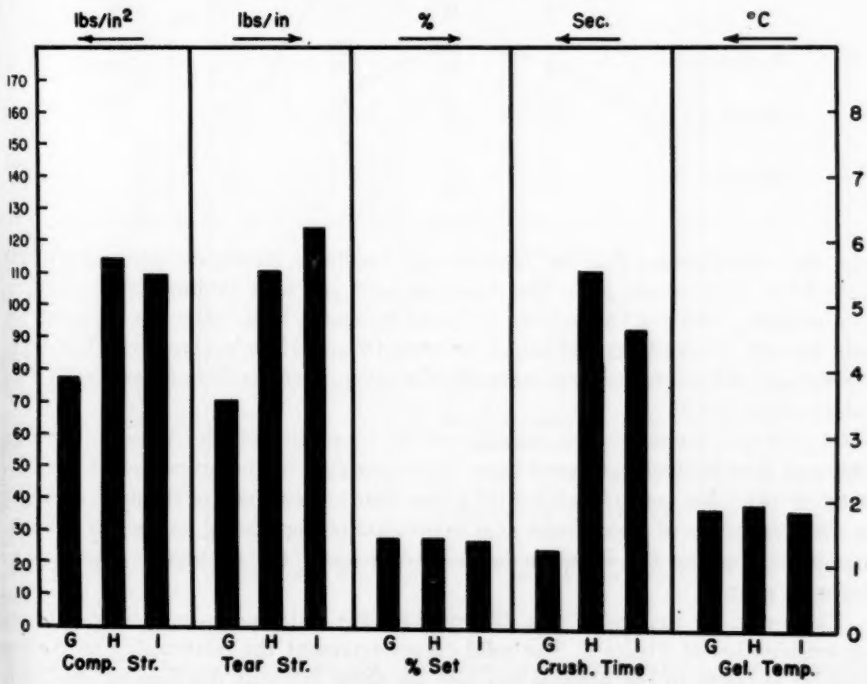

FIG. 4.—Properties of impression compounds

deformation rate of 10 inches/minute, of the duplicating compounds generally ranged between 50 and 65 lb/square inch. Two exceptions to this generalization were compounds B and E. Compound B is a plastic gel and would be expected to have the much higher compressive strength of 130 lb/square inch, and agar compound E is diluted more than the other agar compounds and would be expected to have the lower strength of 37 lb/square inch.

The compressive strength of the agar impression compounds G, H, and I fall between 78 and 114 lb/square inch. These values are higher than for the duplicating compounds, since the agar concentration is two to three times higher than for the duplicating compounds.

The compressive strengths of experimental agar gels listed in Table 3 show considerable variation. The Portuguese, Spanish, and Japanese agar gels showed more variation between batches than did the American agar gels. The Spanish and Portuguese

TABLE 3

PHYSICAL PROPERTIES OF 5 PER CENT COMMERCIAL AGAR GELS

Agar	Batch	Compressive Strength (lb/in²)	Crushing Time (Seconds at 870 gm/cm²)	Per Cent Set	Gel Temperature, (° C.)
American	1	38	26	1.6	36
	2	38	16	2.4	.....
	3	40	19	2.6	37
	4	32	13	2.5	36
	5	36	13	2.4	36
Portuguese	1	60	359	1.5	36
	2	52	134	1.6	36
Spanish	1	70	337	1.6	36
	2	53	53	2.3	36
Japanese	1	27	7	1.7	.....
	2	16	1	3.9	.....

agar gels were stronger than the Japanese and American, having compressive strengths from 52 to 70 lb/square inch. The American agar gels were intermediate in compressive strength, with the values from 32 to 40 lb/square inch, while the Japanese agar gels were the weakest, having values between 16 and 27 lb/square inch. The experimental agar gels contained approximately the same concentration of agar as did duplicating compound E.

Of principal interest is the stability of the properties of the American agar gels prepared from various batches of agar. The variability of the properties of agar from batch to batch has been recognized for a long time as being one of the main difficulties in the preparation of a consistent agar impression or duplicating compound. This fact is substantiated by the variation in the properties of the Portuguese, Spanish, and Japanese agar.

The reason for the choice of a deformation rate of 10 inches/minute may be seen by examination of Figure 5. The solid curves represent the relationship of the compressive strength to the deformation rate for three different dilutions of duplicating

compound A. The same types of curves have been obtained for the other agar duplicating and impression materials, although the maximum is not so pronounced in the case of the impression compounds. The dotted line indicates the per cent compression at fracture of the agar gels prepared by diluting material A with 0.5, 1, or 2 parts of water. All the values fell on a single curve, which showed that within this dilution range the percent compression was independent of the water to material ratio.

The curves for the compressive strength of agar gels versus deformation rate reached a maximum between 10 and 12 inches/minute. If the gels were deformed at low rates, such as 2 inches/minute, or at high rates, such as 20 inches/minute, considerably lower values for compressive strengths were obtained. A maximum was not observed for the per cent compression versus deformation rate, but the values increased between 2 and 10 inches/minute, after which a stable value was maintained. The deformation rate of 10 inches/minute was therefore selected because a maximum value was obtained at this rate and because the materials as used would be subjected to compression forces at approximately this rate.

The significance of selecting the proper deformation rate for evaluating compressive

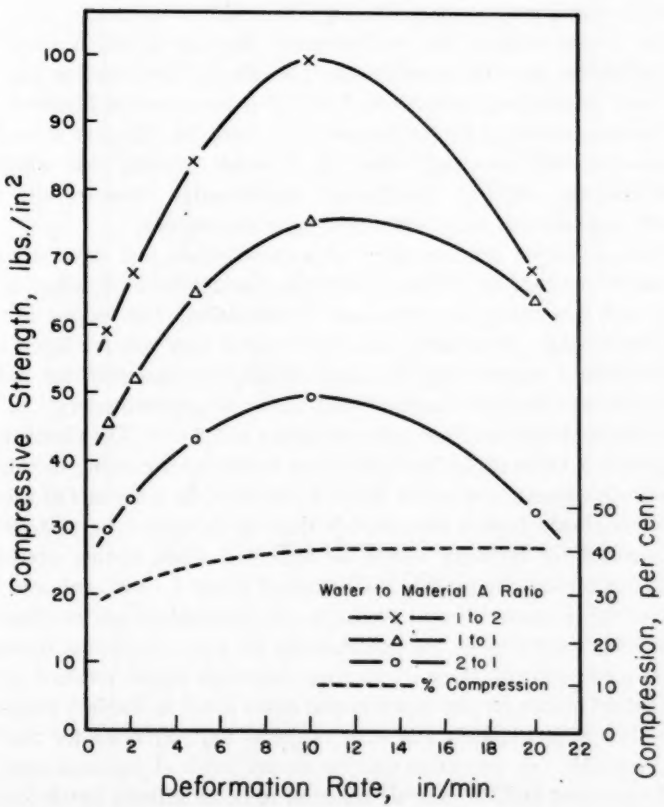

FIG. 5.—The effect of deformation rate on the compressive strength and per cent compression of duplicating material A.

strength may be seen by comparing the values reported by Paffenbarger[2] for agar impression materials with those listed in Figure 4. The values given by Paffenbarger[2] ranged from 16 to 40 lb/square inch, while those reported in this study were between 78 and 114 lb/square inch. The former values are possibly lower because the deformation rate was much slower.

Tear strength.—Since one visual criterion for discarding a duplicating material is the tendency of the material to tear in the region of the interproximal spaces, the tear strength of duplicating compounds is a most important property. The tear specimens were tested in tension at a rate of 10 inches/minute, and the values obtained for the duplicating and impression compounds are shown in Figures 3 and 4.

The order of the tear strengths of the duplicating and impression compounds is essentially the same as for their compressive strengths. The tear-strength test, however, appears to be somewhat more sensitive to differences in materials than the compressive-strength test. The tear strengths of agar duplicating compounds A, D, and F were between 2 and 3 lb/inch, while compounds C and E had lower values of about 1 lb/inch, and the plastic duplicating material had a higher value of over 6 lb/inch. The tear strength of the agar impression compounds were approximately twice the value of the duplicating compounds, having values of 4–6 lb/inch.

In addition to the value of the tear strength, the rate at which a cast is removed from the duplication material is important. The effect of deformation rate on the tear strength of agar duplicating compounds A and D is presented in Figure 6. The results are similar to those obtained for the compressive strengths. The tear strength increased as the deformation rate increased from 1 to 10 inches/minute, after which the values appeared to decrease slightly, though not significantly. These results confirm the recommended procedure of snap removal of agar impressions.

Per cent set.—The per cent set after 12 per cent strain test was run by deforming the specimens 12 per cent for 1 minute, relieving the deformation, allowing a 1-minute rest period, and measuring the permanent deformation. The values for duplicating compounds, impression compounds, and experimental agar gels are listed in Figures 3 and 4 and in Table 3, respectively. The agar duplicating materials that had the higher compressive and tear strengths had per cent values of approximately 1.5. The weaker agar duplicating materials had per cent set values of 2.3–2.9. The plastic gel duplicating material gave a value of 2.6 per cent when evaluated according to this test procedure. There is some question whether this is a good test for materials of this type, since it recovers more slowly from a deformation than do the agar-type materials. A longer time of 5 minutes for recovery would be indicated when testing plastic gels. The stronger agar impression compounds had values of about 1.3 per cent, and little difference was observed between different products. All these values are less than 3 per cent, which is the limit suggested by the specification for agar impression materials. These data indicate that clinically these duplicating materials would function satisfactorily.

The per cent set values for the experimental agars listed in Table 3 were between 1.5 and 2.6, which is approximately the same range as that obtained for the commercial duplicating materials. One exception was the second batch of Japanese agar, which had a value of 3.9 per cent and, in general, appeal to be an inferior batch for dental purposes.

The per cent set test does show large variations in agar gels but does not appear to be a particularly sensitive test. Large increases in the per cent set occur only when the material becomes excessively weak.

It should be pointed out that the per cent set will vary depending on the time allowed for the specimen to recover. The per cent set versus recovery time for duplicating compounds A, B, and E are shown in Figure 7. Material E is normally diluted 3 to 1, but the per cent set versus recovery time curves were determined at 3 to 1 and 1 to 1 dilution, in order to show the effect of agar concentration.

The change in per cent set with recovery time was very slight with duplicating compounds A and E, which were diluted 1 to 1 in their preparation. When material E was used at a 3 to 1 dilution, the per cent set was higher at all comparable recovery times, and the change with time was more pronounced. The change in per cent set with time for the plastic duplicating compound was most pronounced, decreasing from 3.2 per cent at ½ minute to 1.3 per cent at 10 minutes. Since 10 minutes usually elapses between withdrawing the master cast and pouring the refactory cast, the lower value of 1.3 per cent more nearly reflects the behavior of this material in service.

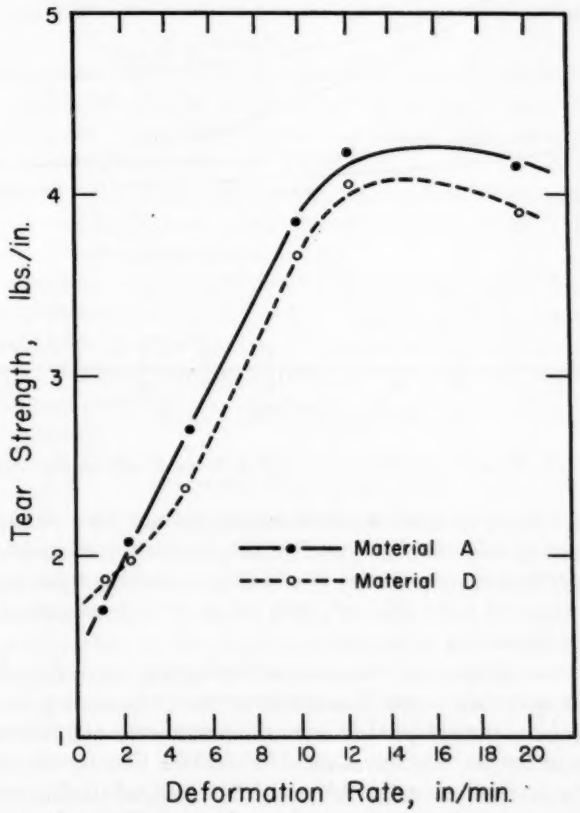

FIG. 6.—The effect of deformation rate on the tear strength of duplicating materials A and D

These data illustrate that the per cent set test, although satisfactory for the evaluation of agar duplicating compounds, may give misleading results when used indiscriminately with other types of materials.

Crushing time.—The crushing-time tests on experimental agars, duplicating compounds, and impression compounds were determined using, 1,100-, 1,600-, and 3,600-gm. loads, respectively. These loads correspond to stress values of 870, 1,265, and 2,845 gm/cm^2, or 12.4, 18.0, and 40.4 lb/square inch. These data are shown in Figures 3 and 4 and Table 3. Unfortunately, it was not possible to use the same load to evaluate both duplicating and impression compounds. The relationship between the two scales is indicated by the fact that the impression compound G, having the lowest crushing time

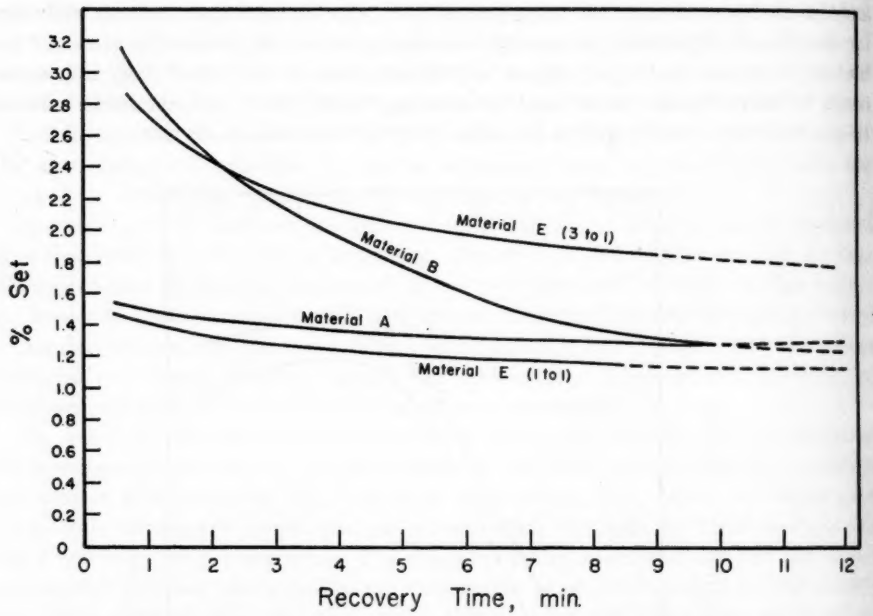

FIG. 7.—The per cent set of duplicating materials A, B, and E with varying recovery times

of 25 seconds at a stress of 2,845 gm/cm^2, had a crushing time of 360 seconds when tested at the stress of 1,265 gm/cm^2 used on the duplicating compounds. Conversely, agar duplicating compounds A, D, and F, which had crushing times of approximately 90 seconds at a stress of 1,265 gm/cm^2, gave values of 1–2 seconds at 2,845 gm/cm^2 stress used to test impression compounds.

The crushing times of about 90 seconds for duplicating compounds A, D, and F, of 10–20 seconds for materials C and E, and greater than 180 seconds for the plastic gel compound B are in the same general order as the compressive and tear strengths and in the inverse order of the per cent set values. The crushing time for the duplicating compound B was also determined at the stress of 2,845 gm/cm^2 used in studying impression compounds, and a time of 50 seconds was obtained. This value was intermediate

to the value of 25 seconds for impression compound G and 90–110 seconds for impression compounds H and I.

The crushing time probably shows differences between materials more clearly than any of the other tests used. This is emphasized by the variation in the times reported in Table 3 for the 5 per cent experimental agar gels. The crushing times, measured at a stress of 870 gm/cm^2, varied from 1 to 359 seconds for batch 2 of the Japanese agar and batch 1 of the Portuguese agar. These data illustrate the variation expected in commercial agars used in the preparation of dental duplicating and impression compounds. It is interesting to note the consistency between the values obtained for the batches of American agars.

The reason why the crushing-time test is so sensitive to changes in elastic duplicating and impression materials is not entirely clear. Unpublished results concerning the aging of duplicating materials confirm these findings, and this test appears to be the most sensitive to changes in materials caused by aging. Study of the elastic, viscoelastic, and permanent deformation properties of these materials has indicated that, with the times required to crush the specimens, the test is evaluating a combination of elastic and viscoelastic properties of these materials under a certain per cent deformation. The compressive strength and per cent set test do not evaluate this combination of qualities.

Gelation temperature.—Of all the properties of agar duplicating and impression materials, the property of gelation temperature was the most constant. The values reported in Figures 3 and 4 and Table 3 show that the gelation temperature varied between 35° and 38° C. according to the A.D.A. specification No. 11 for hydrocolloid impression material—agar type. The galtion temperature versus agar concentration curve must have a very small slope, since a change from a 1:1 to a 3:1 dilution decreases the gelation temperature approximately 1.5° C.

Duplicating qualities.—Any of the duplicating materials tested had elastic properties sufficiently adequate that a rupture-free impression of the master metal model, described in the A.D.A. specification No. 11, section 4.4.3, could be made. The dimensions of stone casts prepared from the impressions were compared with the brass master model. No significant difference was observed in the accuracy of the various duplicating materials evaluated.

SUMMARY AND CONCLUSIONS

The stress-strain properties, compressive strengths, tear strengths, per cent sets after 12 per cent strain, crushing times, gelation temperatures, and duplicating qualities of six duplicating compounds were determined and compared with properties of three agar impression compounds.

The strength properties of the duplicating materials were lower than those of the impression materials as a result of the lower agar concentration.

The duplicating compounds could be divided into three groups with respect to physical properties. The reversible plastic gel had the highest strength properties, the agar duplicating compounds diluted 1–1 with water had intermediate properties, and the agar compounds diluted with 2 or 3 parts of water had the lowest strength properties.

Although differences existed in the strength properties of the various materials, little difference was observed in their gelation temperature or the duplicating qualities, which indicates that a wide variety of materials may be used for duplication procedures.

The stress-strain curves for these materials differed from those previously published. This probably results from the fact that it is now possible to determine the entire stress-strain curve in less than 3 seconds by using improved equipment. The stress-strain properties, therefore, more closely reflect the properties of these materials as used, since rapid removal of the impression or cast is usual in impression or duplicating methods.

The compressive and tear strengths of agar impression or duplicating materials were shown to be a function of deformation rate, the values reaching a maximum value at 10 inches/minute. These data are further substantiation for the practice of a snap removal of impressions or casts.

The time required to rupture a material at a constant stress, or crushing time, was found to be the most sensitive test for evaluating differences in agar materials, probably because it measures both elastic and viscoelastic characteristics.

The per cent set test was found to be a rather insensitive test, but it was able to show large variations in agar material. It was observed that the use of this test with another type of material could give misleading information if the recovery characteristics of the material are not considered.

The two major areas of aging and duplication accuracy, which are important to the evaluation of duplicating compounds, were not discussed but are currently being investigated and will be reported at a later date.

The assistance of G. C. Berry, D. R. Hegg, and R. C. Stanley in the collection of experimental data is gratefully acknowledged.

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Errors and Discrepancies in Measurement of Tooth Size

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The purpose of this study was to evaluate the experimental errors and discrepancies involved in the measurement of tooth size using plaster casts. The terminology employed is taken principally from Beers,[1] who defines the terms used in this area as follows:

A. *Error.* This word is used correctly with two different meanings:
   1. To denote the difference between a measured value and the "true" value. Except in a few trivial cases, the "true" value is unknown and the magnitude of the error is hypothetical.
   2. When a number such as (±.087) is given or implied, "error" refers to the estimated uncertainty in an experiment and is expressed in terms of such quantities as standard deviation, average deviation, probable error, or precision index.

B. *Discrepancy.* This is the difference between two measured values of a quantity, such as the difference between two measured values of the same quantity obtained by two investigators. The word "error" is often used incorrectly to refer to such differences.

C. *Experimental errors.* When a given measurement is repeated, the resulting values, in general, do not agree exactly. The differences between the resulting values are called "experimental errors." Also, they are sometimes called "erratic" or "accidental errors."

D. *Systematic or constant errors.* If, on the other hand, all of the individual values are in error by the same amount, the errors are called *systematic* or *constant* errors.

In the measurement of dental casts, both experimental and systematic errors may be present. Sometimes they arise from the same source. For example, if an investigator uses dividers for the measurement of mesiodistal tooth diameter, the values are apt to be consistently larger than the "true" value because of the taper on the points of the dividers. The true points do not touch the greatest width of the tooth when placed on the tooth, but rather the sides of the points are in contact with the tooth. This error is included in the measure when read off on the scale, since it is the distance between the true points that is read off. The error introduced is thus a *systematic* error. When the tooth is remeasured by the same technique, the points of the dividers may not be placed in exactly the same position on the tooth, and the value read off on the scale will differ from the previous value. This is an *experimental* error.

It should be remembered that the terms "error" and "discrepancy" are used here in their technical sense and do not encompass what is commonly called "mistakes." A reading of 0.87 instead of 0.78 is a mistake, with which no systematic treatment of error could cope.

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When measuring dental casts, as in any investigation, it is obvious that errors and discrepancies will occur. If the data obtained are to be used for comparisons between various population and racial groups or between twins, it is important to know how much of the differences obtained are real differences and how much may be accounted for by errors and discrepancies. Selmer-Olsen[2] suggests that one is in danger of laying more stress on the numerical results than the accuracy of the measuring technique justifies. It follows, then, that the investigator must know what the numerical values of the errors and discrepancies in his methods are before any valid conclusions can be made.

A review of anthropologic and dental literature shows that little mention has been made of errors and discrepancies in the measurement of dental casts, with the notable exception of the Scandinavian investigators such as Selmer-Olsen,[2] Seipel,[3] and Lundstrom.[4] Recently, Moorrees *et al.*[5] gave a detailed account of the discrepancies and errors in their study: "The errors of measurement by different investigators, both of mesiodistal crown diameter of individual permanent teeth and of the combined mesiodistal crown diameters of a series of such teeth ($P_{m_2} - P_{m_2}$), were calculated from a number of double determinations and expressed as standard deviations of the differences. For single permanent mandibular teeth of different tooth classes the error of measurement was 0.09 mm. ($n = 360$) and for the combined mesiodistal crown diameters of ten permanent mandibular ($P_{m_2} - P_{m_2}$) teeth it was 0.41 mm. ($n = 30$)."

In "Variation of Tooth Position," Seipel[3] states: "The exactitude of the measurements depends on the safety of the chosen points, the precision of the measuring instrument, and on the way in which the investigator uses it" (p. 27).

Part I

The purpose of the first portion of this study was to evaluate the errors and discrepancies between two commonly used measuring instruments and between two investigators. The materials used consisted of the plaster casts of 24 subjects chosen randomly from those obtained in 1941 from the University of Michigan Elementary School Growth Study. Impressions were all taken with an alginate material and poured in model plaster.

METHOD

The first method of measurement involved the use of a pair of engineering dividers (with wheel-screw adjustment) and a millimeter scale especially engraved to permit readings in tenths of millimeters. The dividers were adjusted to the mesiodistal diameter of the tooth being measured, and the size was read off from the engraved millimeter scale by putting the points of the dividers on the scale. The other method involved the use of a regular sliding caliper (Helios style) with a Vernier scale as an integral part of the instrument. Both methods permit readings to the nearest 0.1 mm.

Central incisors through first permanent molars in all four quadrants, or 24 teeth, were measured where possible. The changing dentitions eliminated some teeth from the study, as did poorly contoured restorations involving contact areas.

The light source was standardized for all measurements, with a desk-type flourescent lamp and goose-neck tungsten lamp. By reading the instrument in the glare of

the light, the eye, instrument, and light source lay approximately in a straight line, thus reducing errors of parallax to a minimum.

When the scale gave a reading precisely between two 0.1 divisions (i.e., precisely between 7.6 and 7.7), a deck of cards, eight marked with a plus and eight marked with a minus, was consulted to decide whether to read the higher or the lower digit. After sixteen such selections, the deck was reshuffled.

The mesiodistal crown diameter was measured wherever possible, with the points of the dividers or calipers parallel to the long axis of the tooth and on the normal contact areas. The dividers or calipers were inserted from the buccal (labial), with the instrument held at a right angle to the long axis in most cases. However, in some instances the measurements were necessarily made with an insertion from the occlusal (incisal), with the instrument held in a plane parallel to the long axis of the tooth. In cases of linguoversion, a lingual insertion was used. When teeth were rotated, the normal contact areas were chosen. Since the widest portion of a tooth is usually

TABLE 1*

EIGHT MEASUREMENTS ON SAME TOOTH AND DIFFERENCE FROM MEAN OF 8 MEASURES (5.15 MM.)

Measure	Method	Investigator	Actual Size	Difference from Mean	Investigator	Actual Size	Difference from Mean
First.....	Dividers	SH1	5.2	0.05	VL1	5.3	0.15
	S. Cal.	SH1	5.0	− .15	VL1	5.1	− .05
Second.....	Dividers	SH2	5.2	.05	VL2	5.4	.25
	S. Cal.	SH2	5.0	−0.15	VL2	5.0	−0.15

* All measures are in millimeters.

toward the buccal, it follows that the measurement from contact point to contact point is not necessarily the greatest mesiodistal diameter. However, the difference is slight.

To study differences between investigators and between first and second measures by the same investigator (as well as instrument differences), each tooth was measured by each investigator, using both dividers and sliding calipers. The procedure was then repeated; thus each tooth was measured eight times.

Since it was the error or variability, not the actual tooth size, in which we were interested for this study, the effect of tooth size was eliminated by calculating, for each tooth, the mean of the eight measurements and subtracting the mean from each measurement. The measurements for the right mandibular central incisor, case No. 371, are shown in Table 1.

These differences were accumulated for the 24 cases, and a 2×2* analysis of variance was performed for the first measurements and for the second measurements for the right mandibular central incisor. For this tooth, the sliding calipers (both investigators) gave a significantly smaller measurement than did the dividers for both first and second measurements. The difference between investigators (both instruments)

* The authors wish to express their thanks for the assistance given by Professor Paul Dwyer, Department of Mathematics, University of Michigan, in the design of the statistical treatment.

was significant for this tooth for the first measurement, but not for the second.

This type of analysis was repeated for all 24 tooth positions measured.

RESULTS

1. In all tooth positions, the dividers, on the average, gave a significantly larger measurement than did the sliding calipers.

2. The mean size of this difference (mean for all positions and both investigators) was 0.153 ±0.026 mm. (S.E. of the difference).

3. Consideration of first and second measurements revealed that in 17 out of 24 positions the mean of the first measurement was larger than the mean of the second and contained more variation, but only 6 of these 17 were significantly larger. When the means of all the first measurements were pooled and compared with the means of all the second measurements, the reduction in variability was significant at the 0.01 level.

4. Means of differences between investigators were significant for measurements of all 4 cuspids, but not consistently for the other positions. It was felt that a sample of two investigators does not provide a sound basis for any conclusions in this matter.

5. Tests for differences in variances for the 6 anterior teeth as opposed to the 6 posterior teeth were significant, suggesting that a tooth-by-tooth analysis would be interesting.

CONCLUSIONS

1. Use of dividers gave, on the average, a significantly larger measurement of tooth size than did sliding calipers. This is probably due to the taper on the points of the dividers. The true points do not touch the greatest width of the tooth when placed on the tooth, but rather the sides of the points. This error is systematically included in the measure when read on the scale, since it is the distance between the true points that is read off. Therefore, there is both a theoretical and a practical advantage to the sliding caliper, since the measures which result when they are used are closer to the true value for the size of the tooth and are, in addition, more easily obtained.

2. There was some reduction in variation in measures around the mean in each set of eight measures, as we go from first measure to second, suggesting that the measuring technique was perfected to some extent. This improvement was not large but suggests that a training period should be included in any study involving this type of measurement.

3. The training period provided in this study did standardize the techniques of both investigators, so that, aside from the reduction in variability seen in both, they measured with similar accuracy except for the cuspids.

4. Technique may depend upon tooth position, since investigators varied on the cuspid measure and variability as a whole was greater for the posterior teeth.

PART II

The purpose of the second part of these investigations was to compare variances for all teeth individually and to study the accidental and experimental errors between investigators and between two sets of replicate casts and measurements, using the same measuring instrument.

The material used for Part II consisted of replicate sets of plaster casts for the teeth of 23 individuals. The second set of casts were taken in each case, within an hour of the first set.

METHOD

In general, the method was identical with the method used in Part I, except that (1) only sliding calipers were used and (2) two sets of casts were available for each subject. Each cast was measured twice by the two investigators, giving again eight measures for each tooth.

Since the presence of fillings should have negligible effect on a study of discrepancies of measurement, the filled teeth were also measured, giving at least 18 determinations for each tooth position. When more than 18 determinations were possible, these were obtained, and the excess above 18 was deleted randomly down to 18.

Again, the mean of the 8 measures for each tooth was obtained, and the differences from this mean were used in the study of discrepancies.

The statistical treatment of the data was designed to provide a six-way analysis of variance, if the assumptions concerning the homogeneity of the variances were met.

The components of the analysis were maxilla versus mandible, left versus right sides, cast I versus cast II, tooth position, and interactions. The differences from the mean of the 8 measures are the variables being studied.

For tests of differences in variability of tooth position, right and left sides, and maxilla or mandible, an estimate was made for each tooth based on a pooled sum of squares of the eight groups of measurements (8 combinations of cast I, cast II, maxilla, mandible, investigator 1, and investigator 2) with 136 degrees of freedom (8×17).

RESULTS

The findings of this study are summarized in Tables 2, 3, 4, and 5. Table 2, which contains the results of testing the variances of the discrepancies for the left side against those of the right side shows that, although the discrepancies for the left side were more variable than the right side more frequently (10 out of 14 times), this difference was significant only for the maxillary central incisors and for the mandibular central and lateral incisors.

Table 3 summarizes the test of variances of discrepancies for maxilla versus those for the mandible and demonstrates that the variances of the discrepancies for the maxillary lateral incisors and first and second molars are significantly greater than those of their mandibular counterparts. On the other hand, the mandibular left cuspid discrepancy variability is significantly greater than that of the maxillary left cuspid. Essentially, this suggests that the maxillary lateral incisors and molars and mandibular cuspids are harder to measure than the corresponding teeth in the opposite arch. This is probably accounted for by the shape of these teeth.

Table 4 summarizes the results of Bartlett's tests (standard statistical test of within-cell homogeneity) on various groupings of differences within each quadrant. The overall Bartlett's test contraindicated a six-way analysis of variance (the variances were not sufficiently homogeneous). We may, however, arrange the variances of the discrepancies in a comparative fashion, as has been done in Table 4. Any tooth position within parentheses is homogeneous with respect to its variability to any other tooth position

TABLE 2

VARIANCE RATIO TESTS FOR WITHIN-CELL DIFFERENCES IN VARIANCE USING POOLED ESTIMATES OF WITHIN-CELL-VARIANCE

(The Side Having the Greater Variability Is to the Left)

Tooth	Right versus Left					
	Mandible			Maxilla		
		F	Prob.		F	Prob.
I_1	L/R	1.58	0.01	L/R	1.99	0.001
I_2	L/R	2.18	0.001	L/R	1.29	.....
C	L/R	1.18	.....	R/L	1.08	.....
P_1	L/R	1.18	.....	L/R	1.35	.....
P_2	R/L	1.15	.....	L/R	1.04	.....
M_1	L/R	1.36	.....	R/L	1.13	.....
M_2	L/R	1.15	.....	R/L	1.22	.....

TABLE 3

VARIANCE RATIO TEST FOR WITHIN-CELL DIFFERENCE IN VARIANCE USING POOLED ESTIMATES OF WITHIN-CELL VARIANCE

(The Jaw Having the Greater Variability Is to the Left.)

Tooth	Mandible versus Maxilla					
	Left			Right		
		F	Prob.		F	Prob.
I_1	Max/Mand	1.23	.....	Mand/Max	1.02	.....
I_2	Mand/Max	1.03	.....	Max/Mand	1.64	0.01
C	Mand/Max	1.73	0.01	Mand/Max	1.36	.....
P_1	Max/Mand	1.10	.....	Mand/Max	1.04	.....
P_2	Max/Mand	1.14	.....	Mand/Max	1.05	.....
M_1	Max/Mand	1.27	.....	Max/Mand	1.95	0.001
M_2	Max/Mand	1.33	.....	Max/Mand	1.86	0.001

TABLE 4

TOOTH POSITIONS IN ORDER OF INCREASING VARIABILITY OF DISCREPANCY, ACCORDING TO QUADRANT

(The Positions inside Parentheses Had Statistically Homogeneous Variances.)

	Least Variability	Most Variability
Maxillary left	C, (I1, I2, P1, P2),	M1, M2
Maxillary right	(I1, I2, C), (P1, P2),	M1, M2
Mandibular left	(I1, I2, C, P1, P2),	(M1, M2)
Mandibular right	(I1, I2), (C, P1),	(P2, M1), M2

within the same parentheses. In general, the variability of the discrepancies increases from central incisor to second molar.

In lieu of the six-way analysis of variance, 28 three-way analyses of variance were performed (one for each tooth position). Each analysis was a test of the differences of means for investigator, cast, and measure and showed that only investigator differences were significant. The observed differences of means between investigators were calculated and are summarized according to position in Table 5. While 19 out of 28 are statistically significant at the 5 per cent level and 16 at the 1 per cent level, these differences are all (with the exception of the maxillary left second molar difference) less than 0.1 mm. in size. This does not seem to be a difference of practical significance. However, it does illustrate a point of some importance. For example, had one investigator measured the teeth of females and the other the teeth of males and the problem

TABLE 5

SUMMARY OF OBSERVED DIFFERENCES OF MEANS BETWEEN TWO INVESTIGATORS ACCORDING TO TOOTH POSITION

	I1	I2	C	P1	P2	M1	M2	Quadrant Means
Maxillary left	0.074*	0.022	0.036*	0.049*	0.050*	0.043	0.104*	0.054
Maxillary right	.063*	.029*	.031*	.097†	.063*	− .100*	− .015	.024
Mandibular left	.068*	.074*	.011	.053*	.003	.028	.036	.039
Mandibular right	0.081*	0.028*	0.035†	0.025	0.072*	0.029	0.049†	0.046

* Significant at 1 per cent level.
† Significant at 5 per cent level.

been that of sex differences in mesiodistal tooth diameter, a statistically significant sex difference could have been obtained which very well might, in reality, have been an investigator discrepancy.

CONCLUSIONS

1. No significant trends are apparent in the analysis of the variability of discrepancies according to jaw (maxilla versus mandible) or side (right versus left). Any differences found may be termed "experimental errors."

2. As was suspected in Part I, the variability in discrepancies generally increases from central incisor to second molar, but these remain within the acceptable limits of experimental error.

3. Tests of mean differences between investigators, casts, and first and second measures were significant at the 0.1 level only for differences between investigators.

4. With one exception, the differences between investigators were less than 0.1 mm. in size. A difference of this magnitude may be of no practical significance. The total mean difference between investigators was 0.04 mm.

PART III

The purpose of the third part of the study was to compare measurements of tooth size made in the mouth with those made on plaster casts and to compare measurements

of tooth size made on soaped casts with those made on unsoaped casts. Twenty Sophomore dental students with complete permanent dentitions from central incisors through first molars provided the subjects for this investigation.

METHOD

Intraoral measurements were made on the teeth of the twenty subjects using the same sliding caliper, as in the previous studies. The same investigator then measured the teeth on the plaster casts. Both intraoral and cast measures were then repeated. Finally, the casts were soaped and measured again.

RESULTS

A preliminary comparison of the size of the differences between first and second measures on the casts and in the mouth showed that the intraoral differences were

TABLE 6

MEANS OF DIFFERENCES BETWEEN CAST AND INTRAORAL MEASURES WITH STANDARD DEVIATIONS AND *t*-SCORES FOR 40 DOUBLE DETERMINATIONS OF MESIODISTAL TOOTH DIAMETER

	Left				Right			
	Mean Diff. (Mm.)	S.D.	*t*	Prob.	Mean Diff. (Mm.)	S.D.	*t*	Prob.
Maxilla:								
I_1	0.08	0.38	1.40	.....	0.04	0.20	1.27	.....
I_2	− .02	.14	0.89	.....	− .02	.35	0.27	.....
C	.08	.16	3.35	0.01	.10	.35	1.71	.....
P_1	.09	.26	2.13	.05	.08	.26	2.06	0.05
P_2	.16	.29	3.55	.01	.18	.25	4.71	.01
M_1	.37	.32	7.55	.01	.31	.34	5.92	.01
Mandible:								
I_1	− .02	.12	1.30	.....	.0	.39	.....	.....
I_2	− .07	.18	2.39	.05	− .04	.21	1.21	.....
C	.08	.19	2.74	.01	.04	.17	1.61	.....
P_1	.04	.25	1.10	.....	.04	.14	2.13	.05
P_2	.10	.24	2.54	.05	.03	.22	0.90	.....
M_1	0.13	0.33	2.45	0.05	0.23	0.28	5.16	0.01

clearly larger and more variable in the maxilla. In the mandible, however, the size of the mean difference between first and second measures was the same intraorally as on the casts, although the variability of the former was very slightly greater.

The cast measures were consistently larger than the intraoral measures, although in the mandible this constituted a smaller amount than in the maxilla. This difference is approximately 0.1 mm., on the average, with the posterior teeth showing the largest differences, especially in the maxilla.

The second measures were somewhat less variable than the first but showed larger mean differences posteriorly than did the first. Since the variances of first and second measures in the cast versus intraoral comparisons were closely similar, the first and second measures were combined.

Table 6 shows these pooled findings. Note the rather large differences to be found in posterior measures. The average mandibular difference is less than 0.1 mm., and the average maxillary difference is 0.12 mm.

The study of soaped versus non-soaped casts showed slightly larger measures for tooth size on soaped casts. While 10 of the 24 teeth studied were significantly larger, the actual size of the difference was less than 0.08 mm. in all cases, and significance was achieved by small variances rather than by large actual differences.

CONCLUSIONS

1. It is clearly more difficult to measure teeth in the mouth than on plaster casts. However, the differences which resulted from this difficulty were not important for anterior teeth. On the other hand, for second bicuspids and molars the cast measurements were systematically 0.1 mm. larger than the intraoral measurements. This was probably due to the difficulty encountered in establishing the widest mesiodistal diameter, particularly in the maxilla. There would seem, then, to be considerable advantage in making measurements of tooth size on plaster casts rather than in the mouth.

2. The comparison between soaped and non-soaped casts suggests that soaping systematically increases the size of the measurement, probably simply by the addition of a slight film. However, the size of this increase is insignificant as far as individual teeth are concerned.

SUMMARY

This investigation of experimental errors and discrepancies involved in the measurement of the mesiodistal tooth diameter on plaster casts included nine comparisons. These consisted of consideration of differences between first and second measurements, differences between measures made by two investigators, differences between measures made with dividers and with sliding calipers, differences between two sets of replicate casts, differences in measuring maxilla versus mandible and left versus right, differences between tooth types, differences between measures made on casts and in the mouth, and differences between measures made on soaped and unsoaped casts. The mean differences and their variabilities were studied.

Of the nine comparisons, four showed only experimental errors. These were comparisons between two investigators, between two sets of replicate casts, between maxillary and mandibular measures, and between left and right sides.

Certain teeth, such as maxillary molars and lateral incisors and mandibular cuspids, were found to be more difficult to measure for morphologic reasons.

To some extent, the more an investigator measures, the more accurately he does so. Also, the differences between measurements made by two investigators can usually be shown to be statistically significant, although these differences are of small magnitude.

The remaining three comparisons showed systematic differences: measurements made with dividers were approximately 0.1 mm. larger, on the average, than measurements made with sliding calipers; measurements made intraorally of maxillary teeth averaged almost 0.1 mm. smaller than those made on plaster casts of the same teeth; and measurements made of soaped casts were systematically larger, though very slightly so, than measures made on unsoaped casts.

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The Effect of Diet and Parotid Gland Secretions on Dental Caries in Rats

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Hunt and Hoppert had demonstrated that hereditary factors can influence resistance or susceptibility to caries in rats.[1] Many investigators have reported that extirpation of all the major salivary glands results in an increase in caries. The results of these investigations have been reviewed in an earlier paper.[2] Using the strains of rats developed by Hunt and Hoppert, it was shown that caries-resistant rats became more susceptible to caries when the submaxillary and sublingual glands and a portion of the parotid ducts were removed.[2, 3] This suggested that certain hereditary factors for caries resistance were associated with saliva. A further study showed that these factors were probably located in the saliva secreted by the submaxillary and sublingual glands rather than by the parotid glands.[4] However, Schwartz, Resnick, and Shaw proved that parotid gland secretions were important in the caries resistance of rats, provided that a high-sucrose, rather than a coarse-particle, cariogenic diet was used.[5] The purpose of this study was to re-evaluate the contribution of the parotid gland to caries resistance and susceptibility.

EXPERIMENTAL METHODS

Twenty-ninth generation caries-resistant rats developed and maintained by Hunt and Hoppert were kept on a non-cariogenic diet* from birth to about 35 days of age. Littermates were then divided into four experimental groups as follows: group 1—unoperated, given modified Hoppert-Webber-Canniff (HWC) coarse-particle ration,[2] group 2—unoperated, given Harvard high-sucrose 700+ ration;[6, 7] group 3—parotid ducts removed, given ration of group 1; group 4—parotid ducts removed, given ration of group 2. The rats were placed individually in wire-bottom cages and were given food and water ad libitum.

The experiment was prolonged for nearly a year to permit the use of two methods for evaluating caries. The "caries time," or the number of days from the date the rat was placed on the cariogenic diet to the day that caries could be seen with the naked eye, has been described previously.[1, 2] Caries was also scored according to the method of Cox, Dodds, Dixon, and Matuschak.[8] Instead of grinding, the teeth were hemisectioned with a steel saw, as described by Keyes,[9] in order to evaluate lesions deep in the fissures.

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* This diet consists of whole powdered milk, 74 per cent; casein, 10 per cent; dried brewer's yeast, 5 per cent; alfalfa, 10 per cent; and sodium chloride, 1 per cent.

Oral lactobacilli and streptococci were determined by a swab-culture technique using Rogosa SL agar and CVA, respectively. The detailed bacteriologic methods were presented in a previous publication.[2]

RESULTS

The data in Tables 1 and 2 show that caries resistance increased when the parotid secretions were prevented from entering the oral cavity and the rats were maintained on the HWC coarse-particle diet. The caries time increasd from 179.3 to 232.0 days

TABLE 1

EFFECT OF PAROTID DUCT EXCISION ON THE "CARIES TIME" OF RATS FED A COARSE-PARTICLE DIET (HWC) AND A HIGH-SUCROSE DIET (700+)

JAW EVALUATED	DIET	MEAN CARIES TIME (DAYS)*		P†
		Unoperated	Parotid Ducts Excised	
Mandibular.....	HWC	179.3 (13)*	232.0 (12)	<0.02
Mandibular.....	700+	261.5 (11)	200.1 (12)	<0.05
Maxillary.....	HWC	281.6 (13)	331.3 (12)	>0.05
Maxillary.....	700+	329.1 (11)	261.5 (12)	<0.01

* Numbers in parentheses denote rats surviving until they developed caries.
† As determined by *t*-test (see ref. 10).

TABLE 2

EFFECT OF PAROTID DUCT EXCISION ON THE "CARIES SCORE" OF RATS FED A COARSE-PARTICLE DIET (HWC) AND A HIGH-SUCROSE DIET (700+)

JAW EVALUATED	DIET	MEDIAN CARIES SCORE*		P†
		Unoperated	Parotid Ducts Excised	
Mandibular.....	HWC	40.5 (12)	19.5 (12)	<0.02
Mandibular.....	700+	1.0 (10)	20.0 (11)	<0.002
Maxillary.....	HWC	3.0 (12)	0.0 (12)	>0.05
Maxillary.....	700+	0.0 (10)	5.0 (11)	<0.05

* Numbers in parentheses denote rats surviving experiment.
† As determined by Mann-Whitney "U" test (see ref. 11).

for the mandibular molars and from 281.6 to 331.3 for the maxillary molars (Table 1).* The caries score decreased from 40.5 to 19.5 for the mandibular molars and from 3.0 to 0.0 for the maxillary molars (Table 2). A *decrease* in caries score is analogous to an *increase* in caries time. The difference in either caries time or caries score for the maxillary molars is not statistically significant, whereas the difference for the mandibular molars is significant at the 2 per cent level.

* Most of the rats did not develop caries in the maxillary molars, but the caries time was calculated as if the rat had developed caries at the termination of the experiment.

When the rats were given the high-sucrose ration, the caries resistance decreased as a result of removing a portion of the parotid ducts. The caries time decreased from 261.5 to 200.1 days for the mandibular molars and from 329.1 to 261.5 for the maxillary molars (Table 1). The caries score increased from 1.0 to 20.0 for the mandibular molars and from 0.0 to 5.0 for the maxillary molars (Table 2). The differences for both maxillary and mandibular molars are statistically significant.

A two-way analysis of variance for the data on caries time is given in Table 3. This analysis shows that a rat with the parotid secretions interrupted develops caries

TABLE 3

TWO-WAY ANALYSIS OF VARIANCE FOR EFFECTS OF PAROTID SECRETION AND DIETS ON CARIES TIME

TREATMENTS	MANDIBULAR MOLARS		MAXILLARY MOLARS	
	F	*P*	*F*	*P*
Parotid secretion.....	0.10	0.05	0.11	0.05
Diets.....	1.8	0.05	0.14	0.05
Interaction.....	8.2	0.01	10.4	0.01

TABLE 4

POPULATIONS OF LACTOBACILLI AND STREPTOCOCCI IN RESPONSE TO TWO CARIOGENIC DIETS AND PAROTID DUCT EXCISION

GROUP OF BACTERIA	WEEKS AFTER INITIATING EXPERIMENT	NUMBERS OF BACTERIA—MEDIAN VALUE $\times 10^{-3}$			
		On HWC Diet		On 700+Diet	
		Control (13)*	Parotid Ducts Excised (12)	Control (11)	Parotid Ducts Excised (11)
Lactobacilli.....	12	0.6	3.1	0.1	0.1
Lactobacilli.....	24	10.0	0.5	0.7	0.3
Streptococci.....	12	920	1,684	880	960
Streptococci.....	24	1,710	1,865	1,240	1,480

* Numbers in parentheses indicate minimum numbers of rats used for each examination.

at different rates on each of the cariogenic diets. One diet apparently reverses the effect of the other; i.e., an unoperated rat develops caries faster than one with the parotid ducts removed on the HWC diet, but an unoperated rat on the 700+ diet develops caries later than one with the parotid ducts removed.

The data for certain oral acidogenic bacteria are given in Table 4. Neither excision of the parotid ducts nor the two different cariogenic diets produced any significant changes in populations of lactobacilli or streptococci. This conclusion was warranted after subjecting the data to the Kruskal-Wallis analysis of variance, a non-parametric test.[12] Similar data have been presented in a previous publication.[9]

DISCUSSION

The interesting point in this study is that a rat deprived of the secretions from the parotid gland increases in resistance to caries on the HWC diet but decreases in resistance on the 700+ diet. This suggests a caries-resistance factor associated with the parotid gland, which was manifest with the 700+ but not with the HWC ration. Schwartz and co-workers had demonstrated previously that the caries resistance decreased in parotidectomized rats on a high-sucrose diet.[5, 13]

The increase in caries resistance in rats with parotid ducts excised and given the HWC diet has also been reported previously.[4] The explanation for this was a compensatory hypertrophy of the submaxillary and sublingual glands.[4] When the latter two glands were removed, there was a decrease in resistance to caries.[4] When the parotid ducts or glands were removed, there was an increase in weight[5, 13] and in salivary flow of the submaxillary and sublingual glands.[14, 15] Therefore, the glands which contributed to caries resistance increased in function.

Another factor which may explain the increase in resistance to caries for rats given the HWC diet following parotid duct excision is that salivary amylase activity is practically eliminated by this operation.[14] The conversion of starch to simpler carbohydrates by amylase is probably important as a nutrient source for cariogenic microörganisms. When this action is curtailed by removing the parotid ducts, the increase in resistance to caries is a logical consequence.

The production of caries on a high-sucrose diet is probably independent of amylase activity, and therefore amylase should not be considered as either a cariogenic or an anticariogenic factor when the 700+ diet is used.

Investigators have shown previously that caries-resistant rats without parotid secretions showed no change in caries experience when given the HWC diet.[5, 16] The rats used by these investigators were probably at the upper limits of resistance, and any treatment designed to increase their resistance would not become evident. The caries-resistant rats used in this and in a previous experiment of Rosen *et al.*[4] were considerably more susceptible than those used by the previous investigators.

The relative merits of the two different methods of scoring used in this study should be considered. The caries score, as devised by Cox *et al.*,[8] allows for greater precision in judging whether a rat has caries. The data collected by this method should not be analyzed by parametric statistics, because a rat with a score of 10 may not have twice the severity or extent of caries with a score of 5. The caries time is suitable only for detection of gross occlusal fissure lesions. If an experiment is continued for the proper length of time, these lesions are easily detectable. The caries time is a measurement on a ratio scale, and the data can be analyzed by parametric statistical analyses. That is, a rat with a caries time of 100 days takes twice as long to develop caries as a rat with a caries time of 50 days. This method was successfully used by Hunt *et al.* in the development of caries-resistant and caries-susceptible strains of rats.[1]

A correlation analysis of the caries time and caries score of the data presented in Tables 1 and 2 showed that there was a significant correlation between the two methods of scoring. Correlation coefficients of —0.73 and —0.75 were obtained for the

mandibular and maxillary molars, respectively. This indicates that rats which develop caries early will show a greater caries score.

SUMMARY

There was a decrease in caries resistance in Hunt-Hoppert caries-resistant rats fed the Harvard high-sucrose 700+ diet after removal of the parotid ducts. There was an increase in caries resistance in the same strain of rats after removal of the parotid ducts when the rats were given the coarse-particle HWC diet.

No significant changes in populations of oral lactobacilli and streptococci occurred after excising the parotid ducts and giving the rats the two different cariogenic rations.

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The Effects of Citric and Phosphoric Acids on the Teeth

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Dental caries was studied in albino rats which received various amounts of citric or phosphoric acid added to a dry, non-cariogenic food. The composition of this diet was, in percentages, as follows: whole-wheat flour, 34.5; potato flour, 33.0; dried whole milk, 10.0; casein, 6.0; peanut oil, 6.5; brewer's yeast, 5.0; salt mixture (including trace elements), 4.0; cod-liver oil, 0.5; linseed oil, 0.5. The diet was slightly acidogenic; the pH of urine was 5.87 ± 0.03.

Three groups of 68, 19, and 6 animals, respectively, were used. The animals of Group I ranged in age from 6 weeks to 7 months; those in Group II were 3 and 10 months old. The experimental diet was initiated in Groups I and II at weaning. The experimental animals in both groups received either 0.5, 1.0, or 1.5 gm. of phosphoric acid per kilogram of food or 1.5, 4.5, or 12.0 gm. of citric acid per kilogram of food. This 1:3 weight ratio between the acids (1:1.5 molar ratio) was based on the pH and the taste of the aqueous solutions. The animals in Group III were 1 year old at the time of caries evaluation and 2 months old at the time the diet was initiated. The experimental animals in this group received either 75 mg. of phosphoric acid or 312 mg. of citric acid per 10 gm. of the basal ration. Control animals were included in all three groups. Non-decalcified, fuchsin-stained sections of the jaw quadrants were subsequently studied for caries under a microscope (*Intern. Z. Vitaminforsch.*, **26**:235, 1955).

None of the experimental animals showed severe lesions with defects of the enamel continuity in the fissures or approximal surfaces. One of the control animals had a severe lesion in the second upper molar on the distobuccal site, and it appeared to be a smooth-surface lesion. All the fissure abnormalities were confined to a slight staining with fuchsin; whether this condition was pathological or only a sign of not yet fully matured dental tissue was not determined. The approximal lesions were chalky white, with loss of enamel translucency. None of the acid-supplemented groups developed more caries, on the average, than did the controls. However, the molars in rats on the acid-supplemented diets, especially those in animals receiving citric acid in the highest dosages, showed more wear and attrition than did the molars of the control animals. Shallow or nearly absent fissures resulted in the older age groups. It was concluded that the enamel-staining properties were significantly fewer in the two older groups than in the younger groups. No distinct differences were noted in the fissure dentino-enamel scores, but scores of approximal enamel lesions were significantly higher in the 7-month-old group than in the younger animals.

The results in Group II were similar to those for Group I. The enamel scores in the 10-month-old rats were significantly lower than in the 3-month-old group. The differences between the fissure dentino-enamel junction scores for both age groups were not statistically significant, nor were those of the approximal surface attack.

After 1 year, no severe lesions had developed in Group III, in spite of the fact that these animals were started on the non-cariogenic ration when they were 2 months old, the age prior to which they had been fed a cariogenic stock diet known to cause many initial carious lesions in older animals within a 2-month period.

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Organized: New York City, December 10, 1920. Of those at the meeting of organization, the following remain active members: Adolph Berger, Theodor Blum, G. W. Clapp, A. H. Merritt, B. B. Palmer, L. M. Waugh.

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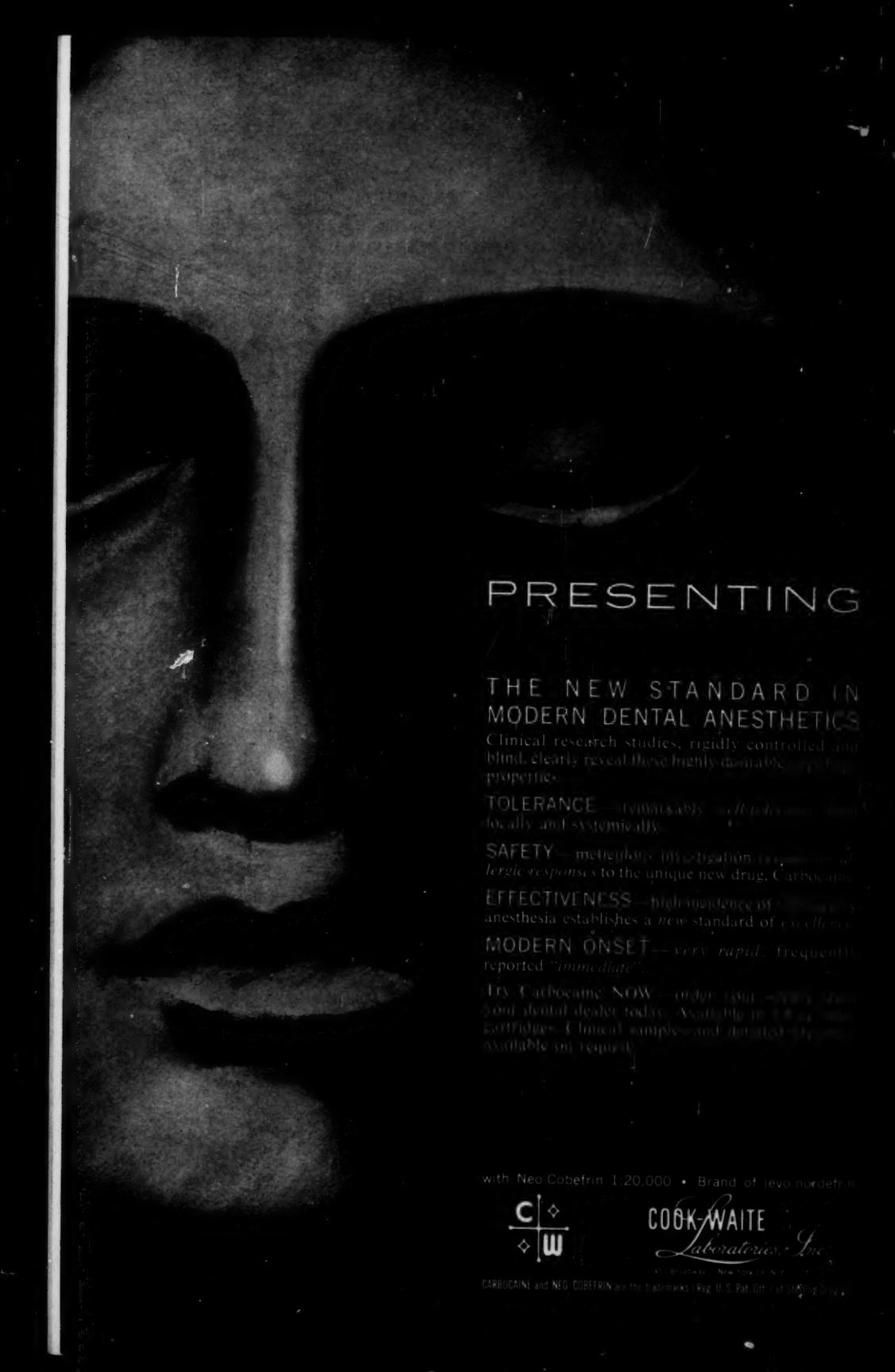
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